RESEARCH NOTES

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IGM and IGG Response to 29–35-kDa *Toxoplasma gondii* Protein Fractions in Experimentally Infected Goats

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ABSTRACT: The evolution of the humoral responses of IgG and IgM against 29–35-kDa *Toxoplasma gondii* fractions from experimentally infected goats were studied and compared by ELISA with the use of whole *T. gondii* soluble extracts and 29–35-kDa electroeluted proteins as antigens. The IgM response to electroeluted proteins was detected from wk 1 to wk 3 postinfection, showing a similar evolution to that observed when *T. gondii* crude extracts were used as antigens. These results suggest that this group of proteins could be used for a more specific detection of anti–*T. gondii* IgM. In the same way, the IgG response was equivalent in both cases, although when 29–35-kDa *T. gondii* fractions were used as antigens, the level of specific IgGs reached a peak 2 wk before than when *T. gondii* crude extract was used. The detection by ELISA of anti–*T. gondii* IgM in goats does not seem to be affected by the presence of specific IgG in serum samples when 29–35-kDa protein fractions were used as antigens.

Toxoplasmosis is one of the most common parasitic diseases in humans and livestock and results in important economic losses due to abortion in small ruminants (Buxton et al., 1998). In many areas, such as the Canary Islands, Spain, the prevalence of *T. gondii* exposure as measured by immunoassay is high in humans (63.35%) and goats (63.30%) (Rodríguez-Ponce et al., 1995, 1999), especially in rural populations, where goats represent an important economic resource and, therefore, a potential role in disease transmission to humans.

Enzyme-linked immunosorbent assay (ELISA) is the most widely used serological method to diagnose toxoplasmosis in domestic animals, including goats (Buxton et al., 1998). However, the protein source used in these tests usually contains multiple *T. gondii* antigens that may increase nonspecific cross-reactivity (Howe et al., 2002; Nishikawa et al., 2002; Dao et al., 2003). Although *T. gondii* target antigens in other species have been studied (Kimbita et al., 2001; Beghetto et al., 2006; Lu et al., 2006), limited information is available in relation to the target antigens that stimulate anti–*T. gondii* IgM and IgG responses in goats. Previous work has shown a specific IgG response to a group of antigens with an apparent molecular weight between 29 and 35 kDa (Conde et al., 2001). The humoral response against a 30-kDa fractions may be the major surface protein of *T. gondii* SAG1 (Conde et al., 2001). The same group of antigens is also recognized by goats infected with different strains of the parasite and sheep vaccinated with the mutant strain S48 (Wastling et al., 1994; Vitor et al., 1999). Moreover, a similar range of antigens, based on molecular weight, is recognized by specific IgG, IgA, and IgM from infected humans by dot-ELISA (Yamamoto et al., 1998).

Specific IgM antibodies are the best indicator of a recent *T. gondii* infection in several species (Payne et al., 1988; Silva et al., 2002), and are complementary to the detection of specific IgG. Together, specific IgM and IgG detection increases the sensitivity of serological tests (Masala et al., 2003). Therefore, the aim of the present study was to determine if *T. gondii* fractions recognized by IgG in infected goats could be useful in the detection of specific IgM and to assess the development of both isotypes throughout the infection. These specific IgG and IgM responses were studied and compared by ELISA with the use of whole *T. gondii* soluble extracts and 29–35-kDa electroeluted proteins as antigens. Finally, we attempted to compare possible competition for the same antigen-binding sites by analyzing complete or IgG-depleted serum. The presence of the parasite in inoculated animals was confirmed by the amplification of a specific fragment of *T. gondii* B1 gene by nested PCR in lymph node samples from infected goats.

In this study, 10, 1-mo-old Canarian goat kids, all seronegative for *T. gondii* by ELISA at the beginning of the experiment, were allocated into 2 groups, i.e., experimentally infected group (I) (n = 6) and control group (C) (n = 4). All animals were housed with unlimited access to commercial pelleted feed and water, and kept under conditions that would prevent incidental *T. gondii* infection. *Toxoplasma gondii*, RH strain, was maintained in 6-wk-old Swiss white mice by serial passage. Tachyzoites (1×10^6) were inoculated i.p., and parasites were harvested from the peritoneum (Conde et al., 2001).

Each goat from group I was inoculated with 2×10^6 *T. gondii* tachyzoites by s.c. injection behind the prescapular lymph node (Uggla and Nilsson, 1987) and compared with the 4 uninfected controls (group C). Both experimental groups of animals were killed at day 90 postinfection (PI), then tissue samples of lymph nodes were frozen in liquid nitrogen and stored at −80 C prior to PCR analysis. Blood samples were collected from the jugular vein to determine antibody levels before inoculation (day 0 PI) and then periodically until day 90 (2, 4, 7, 14, 28, 56, and 90 days PI).

To confirm *T. gondii* infection in inoculated goats, a nested PCR assay was performed on purified DNA from prescapular and mesenteric lymph node samples. A nested-PCR assay was performed using *T. gondii* B1 gene sequences products. The authenticity of the amplified products was confirmed by hybridization with a digoxigenin-labeled B1 gene probe (103 pb fragment) (Alonso et al., 2002). *Toxoplasma gondii* DNA was detected in 1, or both, lymph nodes from all inoculated goats, whereas no PCR products were amplified from the control group.

A range of RH *T. gondii* tachyzoite antigens (approximately 29–35 kDa) previously detected by Western blot immunoassay (Conde et al., 2001) were localized in gels by a colored marked protein and electroeluted with the use of a GE200 SixPac Gel Eluter (Hoefer Scientific Instruments, San Francisco, California), in accordance with the manufacturer's protocols (Ruiz et al., 2003).

To compare the specific IgG and IgM responses against whole *T. gondii* soluble extracts and electroeluted proteins in experimentally infected goats, an indirect ELISA was performed as previously described (Conde et al., 2001; Conde, 2002). Briefly, the antigens were diluted in 0.05 M carbonate–bicarbonate buffer at a final concentration of 25 and 5 μg/ml for IgM and IgG immunoassays, respectively. Bovine serum albumin (BSA) (Sigma, St. Louis, Missouri) was used as a blocking solution, and sera were diluted to 1:100. The level of specific IgM antibody was detected with the use of a primary rabbit antigoat IgM serum (Nordic, Tilburg, the Netherlands) (1:400) and a secondary antirabbit IgG conjugate to peroxidase (Sigma) (1:5,000) as secondary and conjugate antibodies, respectively. Optical densities (O.D.) were measured at 492 nm and the results expressed in relative units (U/ml) based on comparison with a standard curve generated from a positive pool of goat sera that had high levels of *T. gondii*–specific IgM. That standard curve was run in duplicate with the use of serial dilutions (from 1:10 to 1:3,200). Specific IgG levels against *T. gondii* crude extract or electroeluted proteins were detected in the goats with the use of an ELISA method previously described (Conde et al., 2001), and the results were expressed in International Units (I.U./ml) based on the values obtained in a reference assay (800 I.U./ml after comparison with a human reference serum from the WHO that used a 4-layer ELISA procedure) (Calamel et al., 1985).

To determine if anti–*T. gondii* IgG might compete for antigenic sites and possibly inhibit the binding of specific IgM when 29–35-kDa protein fractions are used as antigen, an anti–*T. gondii* IgM ELISA was performed on experimentally infected serum samples that were first treated to remove IgG fractions by affinity chromatography (Chaterton et al., 1999). We confirmed by ELISA IgG that *T. gondii*–specific IgGs were removed by these procedures.

A Student's *t*-test was used to determine the differences between uninfected controls and infected groups through the course of the ex-

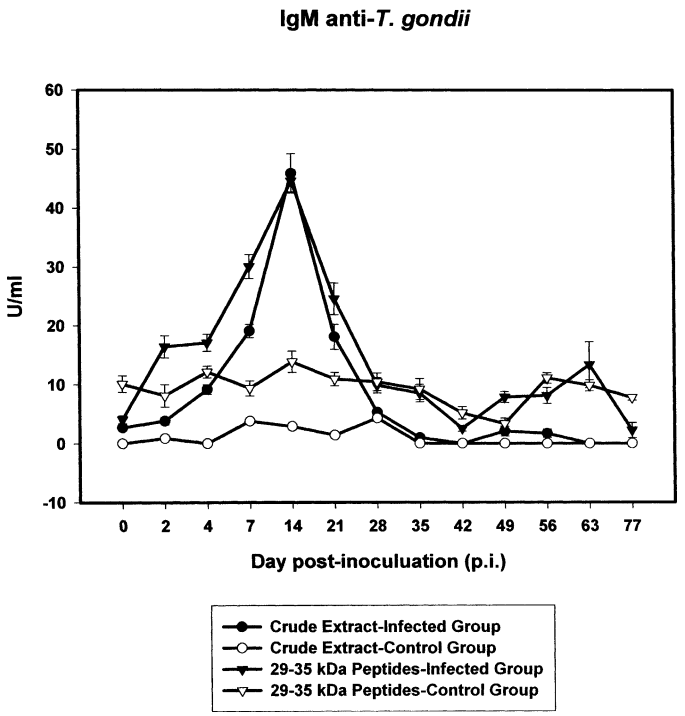

FIGURE 1. ELISA anti–*T. gondii* IgM levels (mean values ± standard error) throughout the experiment in the sera from inoculated and control groups of goats. *Toxoplasma gondii* crude extract or *T. gondii* electroeluted (approximately 29–35 kDa) proteins were used as antigens. Results have been expressed as relative units per ml (U/ml).

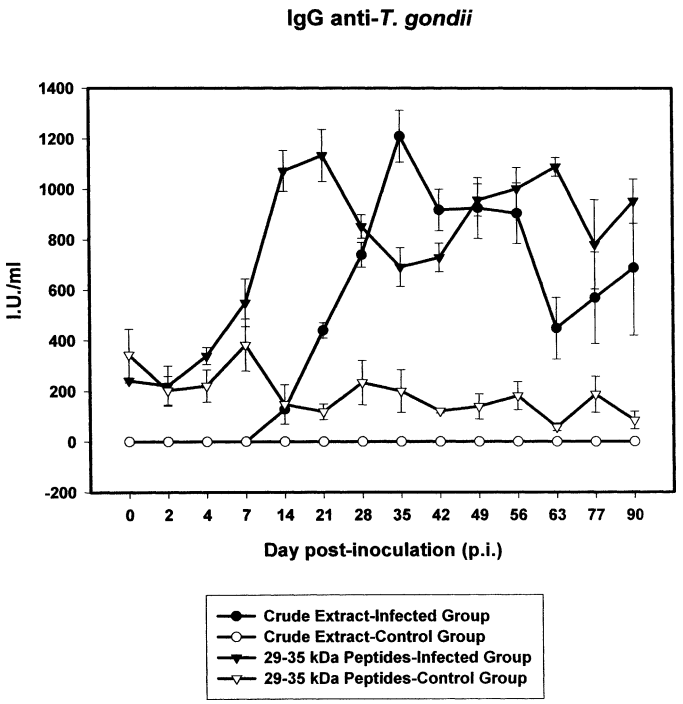

FIGURE 2. ELISA anti–*T. gondii* IgG levels (mean values ± standard error) throughout the experiment in the sera from inoculated and control groups of goats. *Toxoplasma gondii* crude extract or *T. gondii* electroeluted (approximately 29–35 kDa) proteins were used as antigens. Results have been expressed as international units per ml (IU/ml).

periment. P values of 0.05 or lower were considered statistically significant.

Although no severe clinical signs of infection were observed, the inoculation of infective RH *T. gondii* tachyzoites produced detectable IgM- and IgG-specific antibody responses (Figs. 1 and 2). When tachyzoite-soluble extracts were used as antigens (Fig. 1), specific IgM seroconversion was observed at day 7 PI ($P < 0.05$) (18.47 U/ml), and reached a peak value at 14 days PI (44.82 U/ml). This response declined gradually until no significant response was observed (28 PI). Specific IgM antibody response against electroeluted proteins (29–35 kDa) (Fig. 1) showed a similar pattern to that observed when tachyzoite crude extract was used as the antigen, although the mean levels of specific IgM were higher in most of the sampling days. The seroconversion of inoculated goats was observed within the first week PI (30.05 U/ml) and reached a peak at 2 wk PI (44.57 U/ml). This response was unaffected by preabsorbing IgG from serum samples, indicating that the early antibody response of infected goats was primarily due to IgM (Fig. 3).

Antitachyzoite (whole extracts) IgG (Fig. 2) was detected first at 14 days PI ($P < 0.01$) (173.9 IU/ml), peaked at 35 days PI ($P < 0.05$) (1.071 IU/ml), and then persisted through the conclusion of the experiment. The IgG antibodies that recognized the electroluted (approximately 29–35 kDa) tachyzoite antigens were significantly higher at 2 wk PI (1,072.6 IU/ml) ($P < 0.05$), and peaked at 3 wk PI (1,133.6 IU/ml), 2 wk before that recorded for crude extracts. In the control group, specific IgM and IgG titers throughout the experiment were similar to those observed at the beginning of the study.

In general, the serological tests from different host species with the use of a *T. gondii* crude extract as an antigen, detect pathogen-specific IgMs within the first week PI, and specific IgG levels around 2–3 wk PI (Payne et al., 1988; Lundén, 1995; Jungersen et al., 2001). Small differences in the precise timing of seroconversion of absolute peak antibody response could be due to variations in the immune response of the host, the strain of *T. gondii*, the inoculation route, the serological methods used in each case, or even the development of false reactions that could be related with the complexity of the antigen. All these fac-

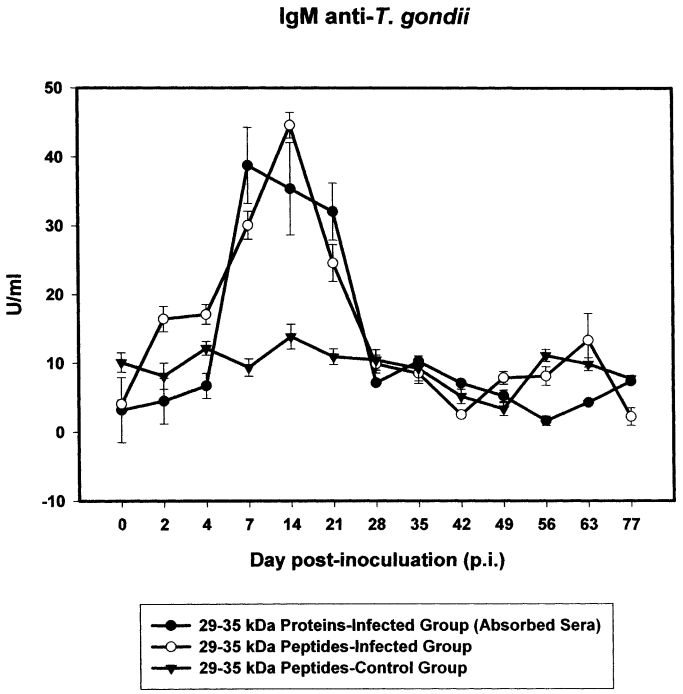

FIGURE 3. ELISA anti–*T. gondii* IgM levels (mean values ± standard error) throughout the experiment in the sera from inoculated (nontreated and absorbed) and control groups of goats. *Toxoplasma gondii* electroeluted (approximately 29–35 kDa) proteins were used as antigens. Results have been expressed as international units per ml (IU/ml).

tors could explain the differences between the results obtained in our study and those observed by other authors (Vitor et al., 1999).

In the present study, the IgM responses to electroeluted proteins were similar to those observed when *T. gondii* crude extract was used as the antigen, suggesting that this group of proteins could be used in the detection of specific IgM, thereby increasing the specificity of the test. This conclusion is supported by an earlier report that demonstrated the use of a reduced number of protein fractions with a molecular weight of approximately 29–35 kDa in the diagnosis of goat toxoplasmosis (Conde et al., 2001).

At same time, the IgG response against this group of antigens showed a similar pattern as when the *T. gondii* crude extract was used as the antigen, but reducing the number of protein fractions that could be responsible for cross-reactions.

In some other species, it has been suggested that a high concentration of specific IgG may reduce detection of specific IgM because of competition for antigen-binding sites (Payne et al., 1988; Lappin et al., 1989). In this study, the immunodepletion of IgG had no effect on the peak IgM response, indicating that detection of *T. gondii*–specific IgM in goats is not substantially inhibited and, thus, it is unnecessary to pretreat serum samples to eliminate cross-reactivity, as introduced in other serological methods (Chaterton et al., 1999).

In conclusion, the IgM and IgG response against the 29–35-kDa parasite antigens could be useful in the diagnosis of both recently acquired and chronic toxoplasmosis in goats, reducing the number of protein fractions that could be involved in the development of cross-reactions. Testing of purified or recombinant proteins within this range of molecular weights under field conditions in naturally infected goats is still needed in order to judge whether these defined antigens might replace current diagnostic methods in goats.

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Microsatellite Markers for the Human Nematode Parasite *Ascaris lumbricoides*: Development and Assessment of Utility

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ABSTRACT: We describe 35 microsatellite markers from the human parasitic nematode *Ascaris lumbricoides*. We found 7 sex-linked markers and demonstrate that 26 autosomal loci can be scored reliably. These markers have high genetic variability and provide the tools to address multiple questions concerning the epidemiology, fine-scale genetic structure, host specificity, and mating systems of this parasite.

Multiple codominant genetic markers provide the most versatile tools for investigating the population structure, mating systems, and host specificity of parasites. However, with the exception of a limited number of polymorphic allozyme loci (Anderson et al., 1993; Ibrahim et al., 1994) and single nucleotide polymorphisms (SNPs) within the introns of nuclear genes (Anderson and Jaenike, 1997), few easily scored codominant loci are available for *Ascaris* spp. Other marker types that have been used include dominant markers such as random amplified polymorphic DNA (RAPD) (Nadler et al., 1995), amplified fragment length polymorphisms (AFLP) (Nejsum, Freydenberg et al., 2005; Nejsum, Parker, et al. 2005), and sequence data from the mitochondrial DNA (mtDNA) or the internal transcribed spacers (ITS) of ribosomal DNA (rDNA) (Anderson et al., 1993, 1995; Peng et al., 1998, 2003, 2005). These markers have provided important insights, but have several limitations. First, for example, population inferences based on mtDNA alone may be misleading, due to processes such as incomplete lineage sorting and introgression via hybridization (Anderson and Jaenike, 1997; Anderson, 2001; Ballard and Whitlock, 2004) or natural selection (Ballard and Whitlock, 2004; Hurst and Jiggins, 2005; Bazin et al., 2006). Second, the low polymorphism and presence of variation both within repeat arrays and between chromosomes complicates interpretation of rDNA ITS data (Blouin, 2002; Vilas et al., 2005). Third, dominant markers (RAPDs and AFLPs) preclude identification of heterozygotes, so deviations from Hardy–Weinberg equilibrium cannot be tested. This latter caveat is important because there may be fitness costs to being inbred (e.g., Christen et al., 2002) and because the change in allele frequency due to directional selection, e.g., antiparasitic drugs, is more rapid in inbred populations (Hedrick, 2005a). Furthermore, sex-linked and autosomal RAPD or AFLP markers cannot be easily differentiated. Treating sex-linked markers as autosomal loci will bias estimates of allele frequencies and can result in incorrect inferences about the genetic structure among populations. Finally, dominant markers are of limited utility in determining mating systems because alleles from both parents cannot be visualized.

The high allelic polymorphism commonly exhibited by microsatellite markers (1–6 bp tandem repeats of DNA sequence) and the ability to genotype individuals at many microsatellite loci allow these markers to be used in a variety of population genetic studies. Such applications include the estimation of inbreeding, migration, relatedness, parentage, effective population size, hybridization, and population assignment (Jarne and Lagoda, 1996; Luikart and England, 1999); and the investigation of mating systems or transmission patterns of parasites among hosts (e.g., Criscione et al., 2005; Criscione and Blouin, 2006). Despite their potential utility, only 5 microsatellite primer pairs have been published to date for *Ascaris* spp. (Anderson et al., 2003).

Here, we present a set of 35 microsatellite markers developed from *A. lumbricoides*. Our objectives in this study were (1) to determine the genotyping reliability of these loci; (2) to characterize the patterns and levels of genetic diversity; and (3) to assess the utility of these loci in detecting roundworm genetic structure among individual human hosts, i.e., infrapopulations (Bush et al., 1997).

The 35 microsatellite loci reported in Table I were generated from 3 sources. We designed primers for 2 sequences that were in GenBank (AF205422 and AF205424). Five primer pairs were designed from sequences obtained from the *A. suum* EST database on Nematode.net (Table I). The remaining 28 were generated from 2 genomic libraries. The 1st library was generated from the combined extracted DNA of 5 Bangladeshi and 5 Guatemalan female worms and the 2nd library was from 4 female Nepali worms. All worms were obtained from human hosts by the methods described in Williams-Blangero et al. (1999, 2002). Protocols for DNA extraction are given in Anderson et al. (2003). Construction of the libraries followed Hamilton et al. (1999). The following biotinylated oligos were used to enrich the libraries: AC_{15}, AG_{15}, $ACCT_{15}$, $AAAC_{15}$, and AAG_{15}. Bacterial colonies were screened with the use of the Phototope-Star Detection Kit from New England Biolabs, Inc. (Ipswich, Massachusetts). Positive colonies were scraped with a pipette tip, which was subsequently immersed in sterile water. These colonies were boiled and then used directly as template for 25-μl PCR reactions (1× PCR buffer, 0.25 mM dNTP, 0.4 μM each primer, 0.25 U TaKaRa Taq™ (Takara Shuzo Co., Otsu, Shiga, Japan), 2.5 mM $MgCl_2$, 1 μl template DNA), with T3 (AATTAACCCTCACT AAAGGG) and T7 (TAATACGACTCACTATAGGG) primers. PCR products were directly sequenced in both directions with the use of a BigDye 3.0 dye terminator sequencing kit (Applied Biosystems, Foster City, California) and T3 and T7 primers. Sequence reaction products were then electrophoresed on an ABI 3100 capillary sequencer. Forward and reverse sequences were compared and aligned with the use of Sequencher V4.2 (Gene Codes Corp., Ann Arbor, Michigan). Oligos for all loci were designed using PRIMER3 (Rozen and Skaletsky, 2000). All primers were ordered from ABI with the tailing option in order to reduce any potential polyadenlyation effects.

We extracted DNA from individual worms as reported previously (Anderson et al., 2003). With female roundworms, we took special care to avoid tissue that potentially can have allelic contamination from male sperm, e.g., uterus (Anderson et al., 2003). Furthermore, any individuals (less than 5% of the data set) that showed 3 or more alleles at any single locus were excluded. Some individuals that had multiple alleles were reextracted. Subsequent PCR showed only 1 or 2 alleles at the suspect locus, thus confirming that sperm contamination was the likely cause (see also Anderson et al., 2003). The PCR reaction mix was as reported in Anderson et al. (2003), but the total volume was scaled down to 5 μl and the final concentration of $MgCl_2$ was 2.5 mM. We used a ramp-down PCR cycling method. There was an initial denaturing step (96 C for 5 min), followed by 5 cycles of high-temperature PCR (96 C for 45 sec, 55 C for 30 sec, 72 C for 1 min), then 35 cycles of low-temperature PCR (96 C for 45 sec, 47 C for 30 sec, 72 C for 1 min), and a final step of 72 C for 7 min. Loci for which cycling conditions varied are shown in Table I. PCR and genotyping error were assessed by duplicating PCRs on a subset of 20 individuals for all loci.

We screened 108 adult *A. lumbricoides* (61 females and 47 males) from 38 human hosts who resided in a single village in Jiri, Nepal, to determine patterns and levels of genetic diversity (Table I). Observed heterozygosity (H_O) and gene diversity (H_E) at each locus were calculated in GENEPOP version 3.4 (Raymond and Rousset, 1995). We found between 2 and 31 alleles per locus, and H_E ranged from 0.12 to 0.95 (Table I). However, for 7 loci (ALAC01, ALGA24, ALGA40,

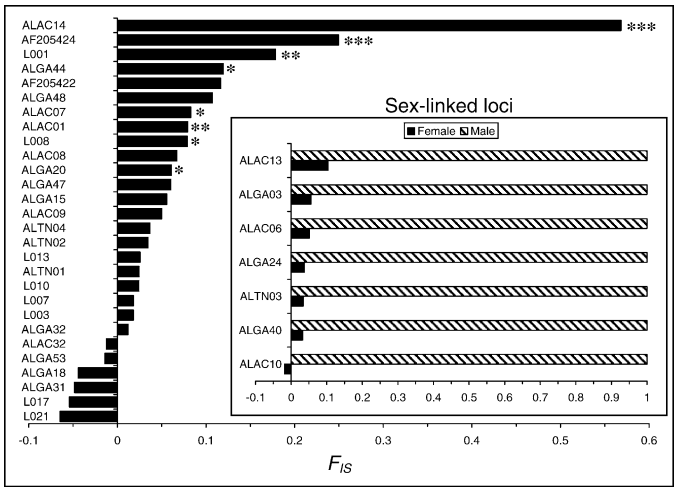

FIGURE 1. F_{IS} for each locus. Negative values indicate heterozygous excess. Positive values indicate homozygous excess. Significance from Hardy–Weinberg equilibrium was determined by permutations of alleles among individuals (*$0.05 \geq P > 0.01$, **$0.01 \geq P > 0.001$, ***$0.001 \geq P$). Inset shows sex-linked loci.

L010, L013, AF205422, and AF205424), the number of alleles is not the true total. Microsatellite stutter for these 7 loci increased in the larger alleles. Therefore, we binned large alleles into a single class for these loci to avoid ambiguity in scoring. PCRs on the duplicate samples always produced the same peaks except for locus AF205424, where the large allele peak heights varied and sometimes failed between duplicates. Therefore, we expected that AF205424 would have an elevated F_{IS} as a result of large allele dropout. In addition, ALAC14 displayed an unusual pattern, where rare alleles were present as homozygotes or with other rare alleles as heterozygotes, but almost never with the single predominate allele (frequency = 0.92). Thus, we also expected an elevated F_{IS} with ALAC 14.

We calculated the Weir and Cockerham (1984) estimator of F_{IS} for each locus with the use of SPAGEDI V1.2 (Hardy and Vekemans, 2002). Positive values of F_{IS} indicate an excess of homozygotes, whereas negative values indicate an excess of heterozygotes from that expected under Hardy–Weinberg equilibrium. We tested for deviations from 0, i.e., a test of Hardy–Weinberg equilibrium, at each locus (and for the multilocus estimate) by randomizing alleles among individuals 20,000 times (Fig. 1). We concluded that 7 loci (ALAC06, ALAC10, ALAC13, ALGA03, ALGA24, ALGA40, and ALTN03) were sex linked because these loci were homozygous in all males ($F_{IS} = 1$), but did not deviate from Hardy–Weinberg equilibrium in female worms (Fig. 1). If sperm contamination was a problem, we would expect that females would be more heterozygous, i.e., have lower F_{IS}, than males across the autosomal loci. However, males had lower F_{IS} in 18 of the 28 autosomal loci, which was not significant (sign test, $P = 0.186$) (data not shown). Of the 28 autosomal loci when males and females were pooled, 8 showed significant ($P \leq 0.05$) heterozygote deficit (Fig. 1). However, only ALAC14 and AF205424 remained significant after sequential Bonferroni correction for multiple testing (Rice, 1989). Furthermore, these were the only 2 autosomal loci to produce significant within host values of F_{IS} in a hierarchical analysis (see below) after sequential Bonferroni correction (data not shown). These results confirmed our a priori expectations that both ALAC 14 and AF205424 would deviate from Hardy–Weinberg equilibrium. Therefore, we considered these 2 loci as outliers and excluded them from all subsequent analyses. In the case of ALAC14, we cannot discriminate between different possible explanations of this pattern, e.g., null alleles, presence of a duplicated locus, or selection; however, it is clear that the F_{IS} of this locus falls well outside the range of values reported for the remaining autosomal loci (Fig. 1). The elevated F_{IS} at locus AF205424 appeared to result from large allele dropout and/or null alleles. The multilocus estimate of F_{IS} based on 26 autosomal loci (excludes ALAC14 and AF205424) was 0.047, demonstrating significant heterozygote deficit ($P < 0.0001$).

The heterozygote deficit detected could result from nonrandom mating within hosts, and/or subdivision between parasites from different hosts, i.e., Wahlund effects. We used a hierarchical analysis of genetic structure (Weir and Cockerham, 1984) to differentiate between these explanations (Table II). For this analysis, we excluded hosts with fewer than 4 genotyped worms, reducing the data set to 66 roundworms from 7 people. We measured both F_{IS} (average within hosts) and F_{ST} (a measure of genetic differentiation among nematode infrapopulations) with the use of FSTAT V2.9.3 (Goudet, 1995). We tested the significance of the average F_{IS} within hosts with the use of 15,000 permutations of alleles among roundworms within hosts. We tested the significance of F_{ST} by the G-based test (Goudet et al., 1996) with 15,000 permutations of individual parasites among hosts. We also determined if the average relatedness (R) (Queller and Goodnight, 1989) among individual roundworms within hosts was significantly different from that expected with random parasite recruitment. For this test, we permuted individuals among hosts 10,000 times with the use of the program SPAGEDI V1.2. We found no significant deviations from Hardy–Weinberg equilibrium within hosts, although there was significant structure among hosts ($F_{ST} > 0$, Table II). These results indicate that the overall F_{IS} observed in our original analysis is caused by the admixture of parasite populations from separate infrapopulations, i.e., a Wahlund effect, rather than nonrandom mating within hosts. Note that the overall F_{IS} (0.047) estimated from the full data set above is approximately equivalent to the F_{IT} (0.041) of this hierarchical analysis, thus confirming that the reduced data set for the hierarchical analysis (66 worms) is representative of the full data that had 108 worms. Furthermore, the average relatedness of roundworms within hosts was greater than expected from a random recruitment of parasites among hosts (Table II). These patterns indicate that hosts may be sampling different source populations of parasites. Thus, the loci we developed appear to be useful in detecting fine scale patterns of genetic structure and parasite transmission.

We tested for genotypic disequilibrium between pairs of loci with the use of GENEPOP (Markov chain parameters: 5,000 dememorizations; 5,000 batches; 5,000 iterations) and used a sequential Bonferroni method to correct for multiple tests. We carried out genotypic disequilibrium tests for the 26 autosomal loci (excluding ALAC14 and AF205424) with combined males and females. We also did these tests with just females across all 33 loci (excluding ALAC14 and AF205424) so as to include the sex-linked loci. After sequential Bonferroni, there were no significant associations between pairs of loci.

Of the 35 microsatellite loci we developed for *A. lumbricoides* (Table I), 2 (AF205424 and ALAC14) are not likely to be useful in future studies. The utility of L021 may also be limited because it has only 2 alleles with a frequency of 0.94 for the common allele. The remaining loci all have substantial genetic variation and, therefore, will be useful for population genetic studies. Preliminary tests (data not shown) also show that all loci will amplify in roundworms obtained from pigs. We note that many of the loci also showed allelic variation due to indels, thus indicating that strict stepwise mutation models are not appropriate for these loci. Caution is needed when comparing levels of microsatellite genetic diversity among species because of differences in isolation protocols and repeat array length of the loci. However, the levels of gene diversity we find in *A. lumbricoides* are similar to that reported in a handful of other parasitic nematodes of animals (reviewed in Johnson et al., 2006). Microsatellites from trichostrongylid nematodes are frequently associated with a ~150-bp repeat element (TcREP-class of repeats) (Grillo et al., 2006; Johnson et al., 2006). We searched our library of clone sequences for homology to the TcREP repeat, but found no BLAST matches.

An interesting result from our microsatellite development was the finding of 7 sex-linked markers. These markers may be useful to help identify the sex of larvae or immature worms. It is noteworthy that *A. suum* has 19 autosomes and 5X chromosomes (2n = 38A + 10X in females, 38A + 5X in males) (Muller and Tobler, 2000). Thus, sex-linked markers should account for ~21% of the genome (assuming all chromosomes are of equal length). In accordance with this estimate, 20% (7 of 35) of our developed markers were sex linked. Interestingly, Johnson et al. (2006) found 3 of 21 (14%) sex-linked microsatellites from *Trichostrongylus tenuis*, which is assumed to have XO sex determination. If similar proportions (14–20%) of dominant markers are lo-

TABLE I. Microsatellite information and measures of genetic diversity (H_O, observed heterozygosity; H_E, gene diversity) from 61 female and 47 male *Ascaris lumbricoides*. Sex-linked markers are in bold.

Locus	GenBank accession	Primers*	Clone size (bp)	Motif	Number of alleles	H_O	H_E
ALAC01	DQ988845	[F]TGCCGGAATTTTATCTTCAA TGATCGACTGTCTATGCAAACC	246	(AC)n	26¶	0.870	0.945
ALAC06†‡	DQ988846	[P]AAAAACATGTGGCTTTGAAT GTCAGTGGTATAGGGCACAT	185	(GT)n	5	0.328	0.345
ALAC07	DQ988847	[P]AATCGTGTTCTTTGAAGTGG AACACGCTGAAATTGAAACT	233	(GT)n	18	0.759	0.828
ALAC08‡§‖	DQ988848	[V]CATTAATTGCAAAGCACAGA CGATTTTGCTGGCTATAGTT	312	(AC)n	25	0.759	0.814
ALAC09	DQ988849	[V]TGCAAATTTTACTATTTTAGCGTTT GATAATTTCATGCCCTACTTGAG	200	(GT)n	14	0.731	0.770
ALAC10†‖	DQ988850	[F]AGATTACGATGTGGCTGACT CGGAACAAATACAAATCCTC	128	(AC)n	5	0.508	0.499
ALAC13†	DQ988851	[F]CTGCAGTCGCGAAAAAGAA TGAAATTCTGTTTCATTCTCAAAA	139	(GT)n	9	0.656	0.731
ALAC14	DQ988852	[P]ACTGAGCAAGGATGCATGTG CAAATGCGAATATGCATGAAA	143	(AC)n	6	0.065	0.150
ALAC32	DQ988853	[F]AACGCTGCCACACCAGTATC ACCTGCGCACATCAAGAC	129	(GT)n	20	0.833	0.823
ALGA03†‖	DQ988854	[V]GCGTGAATGGATCATATTTC CGATAATGCAGATTTGTTGA	196	(TC)n	12	0.639	0.677
ALGA15‡§	DQ988855	[F]TTGGATGTTCTCATCTCATCTC ATGAGGAGYCATCACATTTT	306	(GA)n	15	0.824	0.873
ALGA18	DQ988856	[P]GATGCTGAGGAACAACAGAA TTTCAAGAAGAAGGAAAGTGG	225	(GA)n	9	0.157	0.151
ALGA20	DQ988857	[F]ATTGTTCCGTTGCTGGAAAG TATACCCACCCTTTCGCCTA	234	(GA)n	31	0.880	0.937
ALGA24†	DQ988858	[F]CGAATCAGAGAATGTTTAGCAA AAATGGTKGAATGTGAGAATTT	206	(TC)n	19¶	0.885	0.919
ALGA31	DQ988859	[P]CGCTTTCTTTTAATAACGCATA TGATGCATAAAAAGAAGTGATT	297	(TC)n	16	0.852	0.813
ALGA32	DQ988860	[V]CGTTCGCTCTAAAGAAATCA AAAATAAACAACAGCCTTCCA	184	(TC)n	23	0.889	0.900
ALGA40†‡	DQ988861	[N]AATTGCATCAAATCCTGACA TATTGGTGAGAAGGCGCAAG	283	(GA)n	12¶	0.705	0.728
ALGA44	DQ988862	[P]GCTGGAGACGCAACTAGATA CAACCAATTTTTGCAGATCA	220	(GA)n	9	0.639	0.725
ALGA47‡	DQ988863	[N]AAGTTGGGCTATTTTCCACA AAACGACAATGAACGGAAAT	171	(GA)n	20	0.843	0.896
ALGA48	DQ988864	[N]AGAAATTTTCCAACCGTTTTT TTGTGTTGGTTCCTCATTTG	218	(CT)n(CA)n	12	0.667	0.746
ALGA53	DQ988865	[F]GCGTTGACTAACATAGAGAAAT TGTGAGAATTAATGGGTTGC	217	(GA)n	17	0.324	0.320
ALTN01‡	DQ988866	[P]CGTGACACTCAGACAAATCA TGTTAATTCGATGAAAATGC	240	(CAAA)n	15	0.796	0.816
ALTN02	DQ988867	[N]CAAAACGGCACTATGAAAA ATATCGATTGGCTCATCAAC	249	(TTTG)n	10	0.546	0.566
ALTN03†	DQ988868	[N]TACCACTGAGCAGTCGCATC TACTATTCCGGGGATTGCAT	270	(AC)n(AAAC)n	14	0.787	0.815
ALTN04	DQ988869	[N]TAATTAGTGACAGCCGGAGT TTGGTCGCAAGAGTTAGAAT	193	(TTTG)n	13	0.722	0.750
L007	DQ988870	[P]TAAAAATCAATGCATCAACG AGAGTTTGCATCGAATTTGT	183	(AC)n	10	0.713	0.726
L008‡§	DQ988871	[P]GAGCAGCAATGTTCACTGTA TGGAAAAATATCACGGAAGT	228	(TC)n	19	0.769	0.834
L010	DQ988872	[N]AACGTAATTTTCATGCTGCT AGGACTTGTTTTGACAGTGG	222	(GA)n	19¶	0.870	0.892
L001-est	CB101754 (kl60g02.y1) #	[V]TTCACAGTTGCTGTTTCTTG AGAGAACGTTTCTTATTTCAGC	99	(TA)n	5	0.417	0.507
L003-est‡§	BQ380931 (kk25a05.y1) #	[V]CGACATTTGTCTCTTCGTTT CTCGACACCACATACATCAA	99	(TA)n	4	0.296	0.302

TABLE I. Continued.

Locus	GenBank accession	Primers*	Clone size (bp)	Motif	Number of alleles	H_O	H_E
L013-est‡§	BM033372 (kh55a04.y1) #	[P]GCATAACCGCTGAAGATACT CGAACTGATAACTAGCAGAGAA	189	(TAT)n	10¶	0.769	0.789
L017-est	BQ835581 (kk63e09.y1) #	[F]TGTTTTGAGGTGGTTTTTCT TCATAGGGATGCTTAATGCT	365	(TTGA)n	5	0.380	0.360
L021-est	CB101812 (kl73f08.y1) #	[N]CGGATTGTATGCTCTCTTCT ATCAATTTTTCAATCGGCTA	276	(TTTA)n	2	0.130	0.122
AF205422	AF205422	[F]GACCGCACTGACTTTACAAC CGATGGATAAGATTTTCGTC	245	(AC)n	16¶	0.444	0.503
AF205424	AF205424	[V]GTATCGTCCGCTTAAAAACC ATGGTTTCTCCATCTGGTA	291	(CT)n	19¶	0.648	0.863

* Superscript at the 5′ end of the forward primer shows fluorescent label (F is 6FAM, P is PET, V is VIC, N is NED).
† Sex-linked loci. The number of alleles, H_O, and H_E are based on the 61 females only.
‡ Number of cycles was 38 in the 2nd cycling round.
§ Annealing temperatures were 53 and 45 C.
‖ Reported in Anderson et al. (2003). The forward primer for ALGA03 has been altered by 1 bp on the 5′ end.
EST sample name from Nematode.net Genome Sequencing Center.
¶ Fragments larger than a given size were binned into a single allelic range to avoid stutter-induced ambiguity in genotype scoring.

TABLE II. Genetic structure within (F_{IS}) and among hosts (F_{ST}), and average relatedness (R) within hosts.

	Estimate	Significance
F_{IS} (average within host)	0.015	Not significant
F_{ST} among hosts	0.027 (0.081)*	$P < 0.0001$
R within hosts	0.023	$P < 0.0001$

* Standardized F_{ST} is shown in parentheses. We standardized the Weir and Cockerham (1984) estimate of F_{ST} among infrapopulations by dividing the $F_{ST\text{-estimate}}$ by $F_{ST\text{-max}}$. $F_{ST\text{-max}}$ was calculated by recoding the data to obtain maximum divergence among populations, i.e., no shared alleles among populations (Meirmans, 2006). Within-population heterozygosity can be high with variable loci such as microsatellites; thus the maximum F_{ST} among populations may be much less than 1 (Hedrick, 2005b). Standardization corrects estimates of F_{ST} from multiallelic loci so they are comparable with estimates derived from diallelic loci (Hedrick, 2005b).

cated on the X chromosomes, then analysis methods using dominant markers will be subject to bias. For example, genetic structure studies with dominant markers rely on the assumption of Hardy–Weinberg equilibrium within populations to estimate heterozygote frequencies in the calculation of F_{ST}. However, males will be haploid for the locus under question; thus allele frequency estimates will be incorrect. Therefore, caution is needed when using dominant markers in *Ascaris* spp. and possibly other nematode parasites.

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Abnormal Morphology of an Adult Rocky Mountain Wood Tick, *Dermacentor andersoni* (Acari: Ixodidae)

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ABSTRACT: During a collection of ticks from vegetation in March 2006, a single adult male Rocky Mountain wood tick, *Dermacentor andersoni* (Stiles, 1908), was collected that exhibited unique morphological anomalies, including the absence of a leg on the right side of the body. Coxa IV on the right side also was missing in this specimen. Such teratological changes have not been reported previously for *D. andersoni*.

Several studies have reported cases of morphological abnormalities in several species of ixodid ticks. The most frequent teratological changes are gynandromorphs, i.e., adult ticks exhibiting a combination of male and female morphological characteristics that have arisen as a consequence of the elimination of a maternal sex chromosome (X) from the zygote during embryonic development (Campana-Rouget, 1959a, 1959b; Oliver and Delfin, 1967; Homsher and Yunker, 1981; Labruna et al., 2002). There are, however, a variety of other types of teratological changes, including asymmetrical duplication of reproductive structures and structural deformities of the hypostome, palps, basis capitulum, idiosoma, and legs (Campana-Rouget, 1959b; Sakla et al., 1980; Latif et al., 1988; Buczek et al., 1991; Buczek, 2000; Estrada-Peña, 2001). In a review of the teratology of ticks, Campana-Rouget (1959b) separated the morphological abnormalities of appendages into 2 major categories: schizomélies, e.g., division of the claws tarsus and femur; and meiomélies, the latter of which were further divided into symmélies (fusion of appendages), atrophiés (appendages reduced in size), and ectromélies (the loss of 1 or more legs). The principal causes of morphological abnormalities in ixodid ticks are thought to be somatic or germinal mutations, exposure to chemical agents or environmental stress, and feeding on unusual or sensitized hosts (Campana-Rouget, 1959b; Sakla et al., 1980; Latif et al., 1988; Buczek, 2000). In some instances, teratological changes in ticks have been induced experimentally (Campana-Rouget, 1959b; Oliver and Delfin, 1967; Buczek, 2000). Nonetheless, the occurrence of morphological anomalies in field-collected ticks is relatively infrequent (Sakla et al., 1980; Tovornik, 1987; Guglielmone et al., 1999; Labruna et al., 2002). In this study, we report the discovery of an anomaly in the morphology of an adult male Rocky Mountain wood tick, *Dermacentor andersoni*.

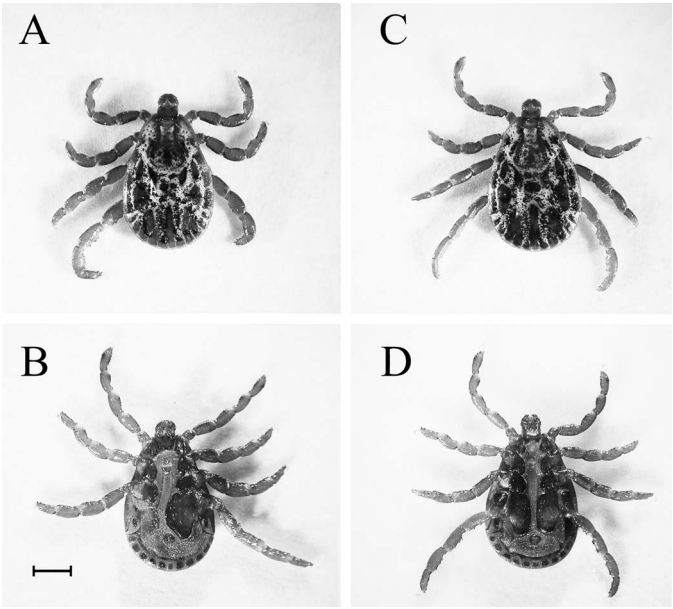

FIGURE 1. *Dermacentor andersoni* males. Dorsal (**A**) and ventral (**B**) views of the abnormal specimen. Dorsal (**C**) and ventral (**D**) views of a normal specimen. Bar = 1 mm.

The abnormal specimen of *D. andersoni* (Fig. 1A, B) was collected by flagging vegetation along walking tracks in Alexander Wilderness Park, Lethbridge, Alberta, Canada (49°44′N, 112°50′W; elevation 840 m). It was 1 of 103 *D. andersoni* adults collected from this location on 18 April 2006. The external morphology of each tick collected was examined using a stereomicroscope, to which a digital camera was attached. The body of the abnormal male tick was 4.3 mm in length (measured from the most anterior point of gnathasoma to the most posterior point of the idiosoma) and 2.6 mm in width (widest point of idiosoma), which was similar to that of many other *D. andersoni* males collected from the same locality. A typical *D. andersoni* male of an equivalent body size (4.4 mm in length and 2.7 mm in width) is shown in Figure 1, C and D, for comparison. The abnormal tick was missing the fourth leg on the right side of the body. Otherwise, the tick seemed normal when viewed dorsally (Fig. 1A). However, an examination of the ventral surface (Fig. 1B) revealed that coxa IV on the right side also was absent. Although both coxae II and III on the right side were similar in size to the corresponding coxae on the left side, they were shaped differently. In addition, the size of the single coxa IV on the abnormal specimen (1.2 × 0.9 mm) was larger than coxa IV of the normal tick (1.0 × 0.6 mm). The larger size of coxa IV on the abnormal tick resulted in the displacement of the median line to the right side of the body. Although the median line was offset from the center of the body, the anus was found approximately midway between the left and right edges of the idiosoma. There were also no differences in the number or size of festoons, or the size or shape of the spiracular plate of the abnormal specimen compared with normal *D. andersoni* males. Despite the morphological anomalies, this abnormal male *D. andersoni* was kept alive in the laboratory at 4 C for at least 130 days. However, it was not determined whether this individual would have had reduced reproductive fitness because of the absence of 1 leg.

Although *D. andersoni* is a common species in regions of western North America (Gregson, 1956; James et al., 2006), the only naturally occurring morphological anomaly previously reported in adults of this species, for which we are aware, has been a single gynandromorph (Homsher and Yunker, 1981). Teratological changes have been induced in *D. andersoni* nymphs due to physical trauma (excision or burning of leg II) at the larval stage (Campana-Rouget, 1959b). Our discovery of a field-collected adult male missing 1 leg and its associated coxa represents the first report of an ectromély in *D. andersoni*. Similar abnormalities have been described in other species of *Dermacentor*, including *D. marginatus*, *D. niveus*, *D. pictus*, and *D. occidentalis* (Campana-Rouget, 1959b; Oliver and Delfin, 1967), and in other tick genera (Feldman-Muhsam, 1950; Campana-Rouget, 1959b; Sakla et al., 1980; Buczek et al., 1991). The detection of only a single abnormal *D. andersoni* in our collection of 103 adults from the Alexander Wilderness Park and an additional 491 adults collected from vegetation at other locations within Alberta and Saskatchewan in Canada suggests that structural malformations in this species are very rare and consistent with the conclusions of other studies (Tovornik, 1987; Guglielmone et al., 1999) that morphological anomalies in ticks are rare phenomena.

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Isolation and Genetic Characterization of *Toxoplasma gondii* From Striped Dolphin (*Stenella coeruleoalba*) From Costa Rica

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ABSTRACT: *Toxoplasma gondii* infection in marine mammals is of interest because of mortality and mode of transmission. It has been suggested that marine mammals become infected with *T. gondii* oocysts washed from land to the sea. We report the isolation and genetic characterization of viable *T. gondii* from a striped dolphin (*Stenella coeruleoalba*), the first time from this host. An adult female dolphin was found stranded on the Pacific Coast of Costa Rica, and the animal died the next day. The dolphin had a high (1:6,400) antibody titer to *T. gondii* in the modified agglutination test. Severe nonsuppurative meningoencephalomyelitis was found in its brain and spinal cord, but *T. gondii* was not found in histological sections of the dolphin. Portions of its brain and the heart were bioassayed in mice for the isolation of *T. gondii*. Viable *T. gondii* was isolated from the brain, but not from the heart, of the dolphin. A cat fed mice infected with the dolphin isolate (designated TgSdCo1) shed oocysts. Genomic DNA from tachyzoites of this isolate was used for genotyping at 10 genetic loci, including SAG1, SAG2, SAG3, BTUB, GRA6, c22-8, c29-2, L358, PK1, and Apico, and this TgSdCo1 isolate was found to be Type II.

Toxoplasma gondii infections are widely prevalent in human beings and other animals worldwide (Dubey and Beattie, 1988). Numerous studies reported the existence of *T. gondii* infections in marine mammals including sea otters, dolphins, seals, and whales (Dubey et al., 2003), and toxoplasmosis has been considered a cause of death in sea otters (Cole et al., 2000; Lindsay, Thomas et al., 2001; Dubey et al., 2003; Kreuder et al., 2003). A toxoplasmosis-like illness was reported in 8 stranded striped dolphins from Spain (Domingo et al., 1992) and Italy (Di Guardo, Agrimi et al., 1995; Di Guardo, Corradi et al., 1995); the diagnosis was based on finding *T. gondii*–like organisms in sections of brain. We report isolation of *T. gondii* from a striped dolphin (*Stenella coeruleoalba*) from Costa Rica, the first time from this host.

An adult female dolphin weighing 58 kg and 210 cm long was found alive, stranded on the Pacific coast of Costa Rica on 9 May 2006, and the animal died the next day. The dolphin was transported to the Departamento de Patología, Escuela Medicina Veterinaria, Universidad Nacional Autonoma, 3,000 Heredia, Costa Rica, where a necropsy examination was performed the same day.

Specimens of tissues were fixed in 10% buffered neutral formalin. For histological studies, paraffin-embedded sections were cut, stained with hematoxylin and eosin, and examined microscopically. Samples of serum, unfixed brain (37 g), and heart (57 g) were forwarded to the Animal Parasitic Diseases Laboratory, Beltsville, Maryland, for parasite examination. Deparaffinized sections of tissues were stained immunohistochemically with *T. gondii* and *Neospora caninum* polyclonal antibodies as described (Lindsay and Dubey, 1989).

Dolphin serum was tested for *T. gondii* antibodies using dilutions from 1:25 to 1:12,800 with the modified agglutination test (MAT) as described by Dubey and Desmonts (1987).

Eight days elapsed between the day of death and bioassay of the dolphin tissues in mice. Brain and heart were homogenized, digested in acid–pepsin (Dubey, 1998), and processed for inoculation into mice. Brain homogenate was inoculated subcutaneously into 5 interferon gamma gene knock out (KO) mice (Dubey and Lindsay, 1998); homogenate of the heart was inoculated subcutaneously into 5 out-bred female Swiss Webster (SW) mice obtained from Taconic Farms, Germantown, New York, as described by Dubey et al. (2002). Tissue imprints of lungs and brain of the mice that died were examined for *T. gondii* tachyzoites or tissue cysts. Survivors were bled on day 49 postinoculation (PI) and a 1:25 dilution of serum from each mouse was tested for *T. gondii* antibodies with the MAT. Mice were killed on day 89 PI, and brains of all mice were examined for tissue cysts as described (Dubey and Beattie, 1988). The inoculated mice were considered infected with *T. gondii* when tachyzoites or tissue cysts were found in tissues.

The 5 KO mice inoculated with the brain of the dolphin died (or killed when moribund) 24 or 25 days PI, and tachyzoites were found in their lungs. Tissues of the 2 KO mice that were killed on day 25 PI were fed to a *T. gondii*–free cat (287); the cat shed oocysts 6 days later. Oocysts were incubated in 2% sulfuric acid for 1 wk at room temperature on a shaker to allow sporulation. Sporulated oocysts were diluted 10-fold and aliquots were inoculated orally into 4 SW mice. The mice that were fed 100–100,000 counted oocysts died of acute toxoplasmosis 7–14 days PI, and tachyzoites were found in their mesenteric lymph nodes or lungs; mice inoculated with tachyzoites of this isolate remained asymptomatic, and tissue cysts were found in their brains 6 wk PI. The mice inoculated with the heart remained asymptomatic, and neither antibodies in their sera nor tissue cysts in their brain were found.

Toxoplasma gondii DNA was extracted from lung tissue of an infected mouse, and strain typing was performed using genetic markers SAG1, SAG2, SAG3, BTUB, GRA6, c22-8, c29-2, L358, PK1, and Apico (Dubey, Sundar et al., 2006; Su et al., 2006) The isolate of *T. gondii* from the striped dolphin was genotype II based on all these markers and was designated TgSdCo1.

The dolphin had a severe nonsuppurative meningoencephalomyelitis, but *T. gondii* was not demonstrable histologically or immunohistochemically in sections of brain and spinal cord; *T. gondii* was not considered as the cause of death of the dolphin.

The ingestion of oocysts in contaminated food or water and the ingestion of *T. gondii*–infected tissues are the 2 main sources of postnatal *T. gondii* infection. The mechanism of *T. gondii* infection in marine mammals is most intriguing because most feed on fish or invertebrates, cold-blooded animals, or they are exclusively herbivorous, thus ingestion of *T. gondii*–infected meat is unlikely. Miller et al. (2002) presented evidence that land-based surface runoff was of significant risk for *T. gondii* infection in sea otters, so it is possible that *T. gondii* oocysts could be washed into the sea via runoff contaminated by cat excrement. The role of marine invertebrates in the life cycle of *T. gondii* is unknown. *Toxoplasma gondii* oocysts are extremely resistant to environmental influences and, therefore, likely to survive in the sea. *Toxoplasma gondii* does not parasitize any cold-blooded animals. However, molluscs can filter large quantities of water and may thus concentrate microbes from the water. Experimentally, *T. gondii* oocysts have been concentrated by mollusks (Lindsay, Phelps et al., 2001; Arkush et al., 2003).

Toxoplasma gondii infection of dolphins is intriguing because they drink little or no water, and their nutritional requirements are derived from fish, squid, or other cold-blooded sea animals that they consume. The prevalence of *T. gondii* antibodies in the bottle-nosed dolphin from the United States is very high (Dubey et al., 2003, 2005), but *T. gondii* has not been isolated from this host.

Among marine mammals, viable *T. gondii* has been isolated from sea otters (Cole et al., 2000; Miller et al., 2001), Pacific harbor seals (Miller et al., 2001), and a California sea lion (Conrad et al., 2005). Based on limited markers, all *T. gondii* sea otter isolates were identified as Type II (Cole et al., 2000). Based on *T. gondii* antigen loci B1, SAG1, SAG2,

SAG3, and GRA6, a new genotype X was proposed for the most of the sea otter *T. gondii* isolates (Miller et al., 2004). Thirty-eight of 50 isolates of *T. gondii* from sea otters from California and the isolate from the harbor seal and the California sea lion, were typed as genotype X, whereas 12 of 50 sea otter isolates were Type II (Conrad et al., 2005), suggesting that the type X genotype predominates in marine mammals in this particular geographical region, which is in contrast to Type II genotype that is widespread in North America and Europe. Our finding of a Type II isolate from dolphin in Costa Rica pacific coastal area suggests that Type II genotype may circulate in a variety of hosts globally.

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Discrimination of *Leishmania braziliensis* Variants by kDNA Signatures Produced by LSSP-PCR

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ABSTRACT: The conventional methods for identification and typing of *Leishmania* species depend on previous culture isolation of the parasites. Not infrequently, culture is unsuccessful and may result in misrepresentation of the heterogeneity of the original isolate. Thus, more reliable and precise identification of genotypes of *Leishmania* spp. is important for a better clinical and epidemiological understanding of the disease. We evaluated the potential of LSSP-PCR targeting kDNA minicircles in discriminating different variants of the parasite with the use of clinical samples directly or cultivated parasites. The 1st step of this procedure consists of the amplification of the minicircles by conventional PCR; the 2nd step is low-stringency amplification of the minicircles previously amplified, with the use of 1 of the primers. Although LSSP-PCR produced complex and distinct kDNA signatures for isolates representing different species, further experiments demonstrated that the approach had the potential for discriminating intraspecific variants of *L. braziliensis*. Thus, the generated profiles were too variable to be useful as markers for species identification. Moreover, we demonstrated that the approach can be directly applied to clinical samples. In conclusion, LSSP-PCR targeting kDNA minicircles produces profiles that reflect polymorphisms of the predominant classes of minicircles, and can be useful for studies aimed at discriminating *Leishmania braziliensis* genotypes without the need for previous cultivation of the parasite.

Leishmaniasis is a disease in vertebrate animals caused by species of *Leishmania* vectored by infected phlebotomine sand flies. These parasites cause a variety of clinical syndromes, including cutaneous or mucocutaneous lesions, as well as visceral forms of disease. Leishmaniasis is endemic in 88 countries on 4 continents. More than 90% of cutaneous leishmaniasis cases occur in Iran, Afghanistan, Syria, Saudi Arabia, Brazil, and Peru. Greater than 90% of visceral leishmaniasis cases occur in Bangladesh, Brazil, India, and Sudan (WHO, 2006).

Several procedures for the diagnosis and identification of species and subspecies of *Leishmania* have been devised, such as analysis of isoenzymes by electrophoresis (Cupolillo et al., 1994), immunological methods (Shaw et al., 1989), and PCR-derived techniques such as RAPD (random amplified polymorphic DNA) (Guizani et al., 2002; Martinez et al., 2003) and SSR-PCR (simple-sequence repeat-anchored PCR) (Volpini et al., 2001). The conventional methods for identification and typing of *Leishmania* species, however, depend on previous culture isolation of the parasites. Not infrequently, culture is unsuccessful and may result in misrepresentation of the heterogeneity of the original isolate. LSSP-PCR (low-stringency single specific primer PCR) is particularly appropriate for the identification of genetic variability directly in clinical samples, including polymorphisms of minicircles of kDNA in *Trypanosoma cruzi* (Vago et al., 1996). The production of kDNA signatures by LSSP-PCR is a procedure in which the 1st step consists of the amplification of the minicircles by conventional PCR, whose specificity depends on the subset of primers used. The 2nd step is low-stringency amplification of the minicircles previously amplified, with the use of 1 of the primers to generate a band profile dependent on the heterogeneity of the target. In the present communication, we show that kDNA signatures produced by LSSP-PCR are able to identify different genotypes of *Leishmania braziliensis*.

The *Leishmania* spp. DNA purification from cultivated parasites or clinical samples was performed with the use of the Genomic Prep Cells and Tissue DNA Isolation Kit® (Amersham Pharmacia Biotech, Uppsala, Sweden), following instructions of the supplier. Conventional PCR was performed in a volume of 100 μl, containing 10 mM Tris-HCl, 50 mM KCl, 0.1 mg/ml gelatin, 1.5 mM $MgCl_2$, 0.2 mM dNTP, 100 pmoles of each primer, and 2.5 U of Taq DNA polymerase.

Parasite isolates corresponding to 9 different species of *Leishmania* were assayed, i.e., *L. chagasi* (MHOM/BR/1974/PP 75), *L. guyanensis* (MHOM/BR/1975/M4147), *L. panamensis* (MHOM/PA/1971/L5 94), *L. mexicana* (MHOM/BZ/1982/BEL 21), *L. major* (MRHO/SU/1959/P-strain), *L. tropica* (MHOM/SU/1958/Strain OD), *L. donovani* (MHOM/ET/1967/HU3), *L. amazonensis* (IFLA/BR/1967/PH 8), and 4 isolates of *L. braziliensis* (MHOM/BR/1975/M2903, MHOM/BR/2002/OLC, MHOM/BR/2002/GBS, MNEC/BR/2003/CPqAM191). Their minicircles were amplified with the pair of primers LSUC (5′-ACAATTAGG GGTTGGTGTAA-3′) and LCUL (5′-TTTTGAACGGGGTTTCTG-3′), which can anneal to kDNA of all species of *Leishmania* (Bhattacharya et al., 1996). Then, LSSP-PCR was performed as reported previously (Pena and Simpson, 1996). Briefly, the reaction was carried out in 25 μl containing 10 mM Tris-HCl, 50 mM KCl, 0.1 mg/ml gelatin, 1.5 mM $MgCl_2$, 0.2 mM dNTP, 100 pmoles of 1 of the primers used in the conventional amplification, 2.5 U of Taq DNA polymerase, and 38 ng of DNA. The cycling conditions consisted of 95 C for 5 min, followed by 40 cycles (95 C for 1 min, 30 C for 1 min, 72 C for 1 min), and a final extension at 72 C for 5 min. LSSP-PCR products were analyzed by vertical 5% polyacrylamide gel electrophoresis stained with the Plus One Silver Staining Kit (Amersham Pharmacia Biotech), according to instructions provided by the supplier. To test for reproducibility, all reactions were performed in at least 2 independent experiments.

LSSP-PCR produced complex and distinct kDNA signatures for the 9 isolates representing different species (data not shown). Even when similar kDNA patterns were obtained with a given primer, the other primer was useful in the final discriminating analysis. This could be observed for the isolates of *L. mexicana* and *L. tropica* that produced similar profiles with primer LSUL and distinct profiles with primer LSUC (data not shown).

kDNA minicircles from *Leishmania* species, which are present at thousands of copies per cell, are about 1 kb in size, contain 1 conserved region of about 250 bp in size, and a single guide RNA (gRNA) gene about 70 bp in size (Brewster and Barker, 2002). Minicircles form a heterogeneous group of DNA sequences comprising several classes, whose number has been explained by the different minicircle-encoded gRNA required by RNA editing (Thiemann et al., 1994; Brewster and Barker, 2002). The number of different classes in any single kinetoplast varies depending on the species. Variation in the apparent number of classes and the frequency of each class is so high that the analysis of kDNA polymorphism can be used to characterize isolates of the parasite. It is assumed that LSSP-PCR targeting kDNA minicircles produce profiles that reflect polymorphisms of the predominant classes of minicircles in each *Leishmania braziliensis* genotype. To determine if LSSP-PCR would be able to discriminate intraspecific variants, 4 *L. braziliensis* isolates were analyzed. Two patterns were similar and 2 were distinct, suggesting that the approach had the potential for discriminating intra-specific variants of the parasite (Fig. 1), but was too variable to be used as markers of species identification. The major advantage of LSSP-PCR is that previous cultivation of the parasite is not necessary, avoiding the risks of misrepresentation of the heterogeneity of the original isolate. On the other hand, it is noteworthy that, depending on the specificity of the primers used in the 1st step of the LSSP-PCR, it is possible to identify genus, subgenus, or some species of the parasite (de Bruijn and Barker, 1992; Bhattacharya et al., 1996; Rodriguez et al., 2000). However, as far as we know, there is no validated PCR system targeting kDNA for the specific detection of *L. bra-*

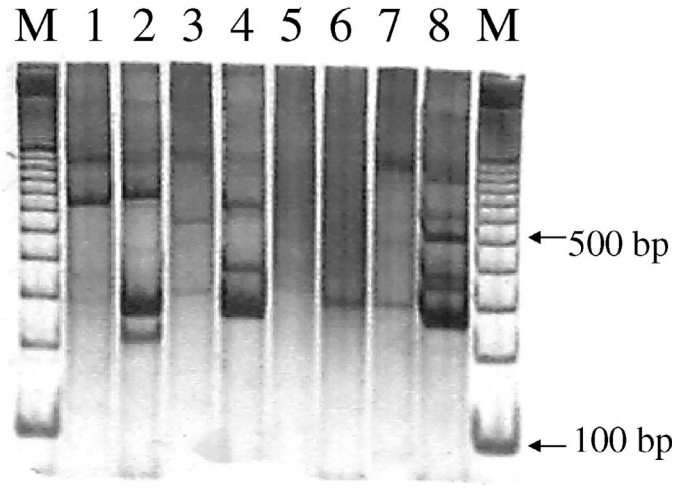

FIGURE 1. Polyacrylamide gel electrophoresis showing the LSSP-PCR profiles of minicircles of 4 *Leishmania braziliensis* isolates. Lanes 1 and 2, isolate 1; lanes 3 and 4, isolate 2; lanes 5 and 6, isolate 3; lanes 7 and 8, isolate 4. Even lanes, primers B1; odd lanes, primer B2. M, molecular-weight marker (100 bp DNA ladder).

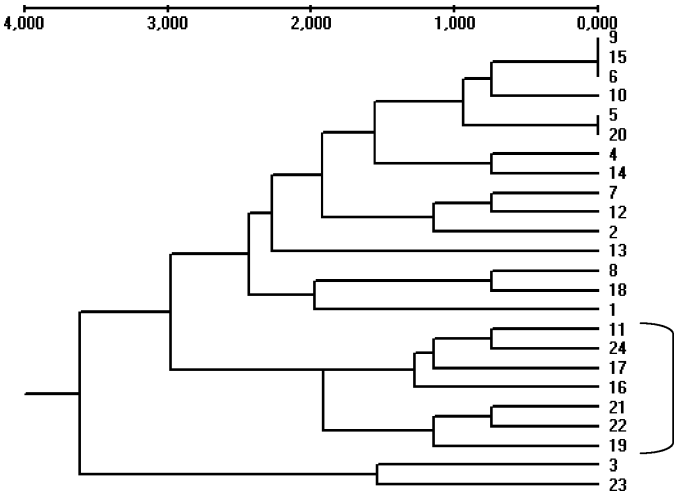

FIGURE 2. Phenogram of LSSP-PCR band profiles generated from minicircles amplified from active lesions (1–12) and scars (13–24) of ACL. The cluster containing 50% of the scar samples is indicated.

ziliensis (Castilho et al., 2003). Thus, in the present communication, we used primers that could detect all *Leishmania* species, and primers specific for the subgenus *Viannia*.

To evaluate the potential for discriminating intraspecific variants further, and to demonstrate that the approach can be directly applied to clinical samples, 12 skin biopsies of lesions from patients with American cutaneous leishmaniasis (ACL), and 12 biopsies of scars from patients clinically cured from the disease were analyzed. These clinical samples were obtained from patients living in the municipality of Amaraji, Pernambuco, Brazil, which is endemic for ACL caused by *L. braziliensis*. The parasite was isolated and characterized from 4 patients with ACL, confirming infection with *L. braziliensis* on the basis of analysis with monoclonal antibodies and isoenzyme analysis. Previous studies had shown that the species of *Leishmania* from patients with ACL and rodents in areas of endemicity in Pernambuco, Brazil were *L. braziliensis* (Brito et al., 1993; Rodrigues et al., 2002; Brandão-Filho et al., 2003; Cupolillo et al., 2003; Mendonca et al., 2004; Andrade et al., 2005). Thus, the evidence available at present incriminates *L. braziliensis* as the only causative agent of ACL in Pernambuco, Brazil. The clinical samples were amplified with primers B1 (5′-GGGGTTGGTG TAATATAGTGG-3′) and B2 (5′-CTAATTGTGCACGGGGAGG-3′), specific for the subgenus *Viannia* (de Bruijn and Barker, 1992), following a previously described protocol (Rodrigues et al., 2002). A phenetic tree was constructed based on band sharing between all possible pairs with the use of the software TFPGA (Tools for Population Genetic Analysis), developed by Mark P. Miller (*Department of Biology, 5305 Old Main Hill, Utah State University, Logan, Utah 84322-5305, e-mail: mark.miller@usu.edu*). This was performed with LSSP-PCR profiles obtained with the use of primer B2 of amplicons derived from active lesions (Fig. 2, samples 1–12) and from scars (Fig. 2, samples 13–24) of ACL. Six of 12 (50%) amplicons derived from scars were grouped in a cluster ($\chi^2 = 5.042$, $P = 0.025$), suggesting that the minicircles of persistent parasites are significantly less diverse. This particular finding was independently confirmed by sequencing of clones of whole minicircles amplified from these clinical samples (data not shown). These findings may suggest that, during the process of healing, there is selection of certain minicircle classes, possibly encoding gRNAs required for RNA editing of genes that are essential for the persistence of the parasites in the scar tissue.

This method has been also used in the identification and differentiation of microorganisms with great genetic variability (van Belkum, 1995; Gomes et al., 1997) and in the study of kDNA of different *T. cruzi* strains (Vago et al., 1996; Andrade et al., 1999). In leishmaniasis, even when the causative agent is already identified, typing is relevant for epidemiological and control purposes, to determine the modes of transmission and the sources of infection. In endemic areas, genotyping is useful in distinguishing between reinfections from reactivation of latent infections (Morales et al., 2001). In addition, with the use of LSSP-PCR and other typing techniques, it may be possible to determine if there is clonal selection during the process of healing of ACL, thereby contributing to our understanding of the factors leading to parasite persistence in leishmaniasis. In conclusion, LSSP-PCR of kDNA minicircles can be useful for epidemiological studies aimed at discriminating variants of *L. braziliensis* without the need for previous cultivation of the parasite.

We thank Elisa Cupolillo (Reference Service for Isolation and Typing of *Leishmania*, Instituto Oswaldo Cruz—FIOCRUZ) for providing us with reference isolates of *Leishmania*, and Plinio Pereira Gomes, Jr., for technical advice. Financial support for this work was provided by FIOCRUZ and CNPq.

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Gregarina niphandrodes (Eugregarinorida: Septatorina): Oocyst Surface Architecture

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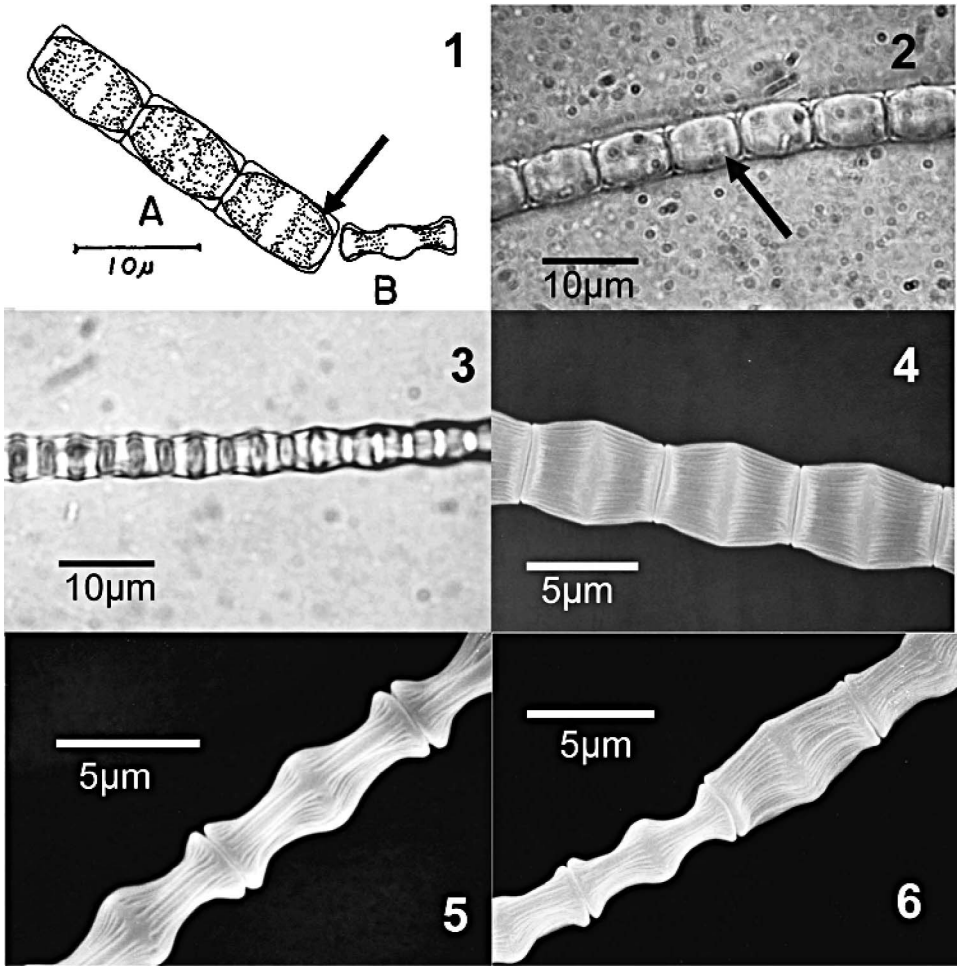
ABSTRACT: The surface architecture of oocysts produced by *Gregarina niphandrodes* (Eugregarinorida) from *Tenebrio molitor* adults (Coleoptera: Tenebrionidae) as revealed by scanning electron microscopy is reported. Gametocysts were allowed to dehisce on 15-mm, round cover glasses; the cover glasses with their oocysts chains were then mounted on stubs without further processing, and sputter-coated with 20-nm gold–palladium. Scanning electron microscopy was performed at 10–15 kV with a Hitachi 3000N SEM. Oocysts retained their characteristic shapes as reported in the original species description but showed longitudinal ridges of relatively uniform height, width, and spacing, in separate fields on either side of a central equatorial bulge in the oocysts. There was no ultrastructural evidence of an enclosing external sheath holding the oocysts in a chain. Oocyst ends were flared slightly, and the chain itself was twisted, with adjacent oocysts offset slightly from one another. This article now provides an additional set of structural characters potentially useful in gregarine systematics.

The class Gregarinasina (Apicomplexa) is perhaps the most diverse eukaryotic taxon because its members parasitize invertebrates, especially annelids and arthropods, although gregarine species have been described from most invertebrate phyla. Descriptive work, however, is somewhat hindered by a number of factors, such as gregarines' lack of medical importance, their seeming paucity of structural features, and the fact that so many potential hosts in interesting parts of the world are relatively inaccessible to workers because of logistical constraints, political turmoil, and lack of funds. Nevertheless, in recent years, Clopton and various coworkers have established criteria by which new taxa should be described and have standardized terminology for shapes and proportions (Clopton et al., 1991, 1992; Clopton, 2004; Clopton et al., 2004). This body of published work shows clearly that oocyst shape and size are highly stable characters of major taxonomic importance.

The present study was intended to increase the number of characters available for gregarine systematics by examining surface architecture of oocysts using scanning electron microscopy. *Gregarina niphandrodes* Clopton, Percival, and Janovy, 1991, from adult *Tenebrio molitor* (Coleoptera: Tenebrionidae) was chosen as the material because both host and parasite are common, well-studied species, and adult beetles are generally infected with only this single gregarine species (Clopton et al., 1992). In addition, *G. niphandrodes* gametocysts are relatively large and easily handled, and oocyst production (''sporogeny'') is easily achieved in moist chambers (Clopton and Janovy, 1993).

Insects used in this study were from cultures maintained for many years at the University of Nebraska–Lincoln using wheat bran and potatoes as food. These are the same cultures from which the type hosts



FIGURES 1–6. (**1**) Drawing of *Gregarina niphandrodes* oocysts from the original species description (Clopton et al., 1991) showing characteristic morphology as seen in 1:1 glycerin:water suspensions. Arrow points to patterns that can now be interpreted as longitudinal ridges. (**2**) Bright-field light micrograph of a *G. niphandrodes* oocyst chain suspended in 1:1 glycerin:water. Arrow points to an area that can now be interpreted as showing longitudinal ridges. (**3**) Bright-field light micrograph of the same *G. niphandrodes* oocyst chain shown in Figure 2, but taken before submersion in glycerin:water. (**4–6**) Scanning electron micrographs of *G. niphandrodes* oocysts, showing surface ridges and the nature of joints between oocysts.

and parasite type specimens were taken (Clopton et al., 1991). Gametocysts were harvested by isolating adult beetles overnight in a plastic shoebox with a slightly moistened paper towel. Shed gametocysts were picked up with a fine camel hair brush and transferred to 1% neutral buffered formalin to be washed for a few minutes before isolation for dehiscence. Gametocysts were placed on 15-mm-diameter, circular cover glasses coated with poly-L-lysine (Sigma, St. Louis, Missouri) according to package instructions and placed in the center well of covered Falcon plastic organ-culture dishes with water in the moat; dehiscence occurred within 3 days. Oocysts were prepared for scanning electron microscopy by simply attaching the cover glass, with its oocyst chains, to a stub then sputter-coating the specimen with 200-nm gold–palladium using a Technics Hummer II sputterer. Scanning electron microscopy was performed at 10–15 kV with a Hitachi 3000N SEM.

Oocysts also were prepared for light microscopy by placing oocyst chains dry on a slide and covering them with a 22 × 22 mm No. 1 cover glass, tacked down at the corners with Elmer's glue. This kind of preparation allowed photographs to be taken of oocysts under oil immersion but as they occur ''naturally,'' i.e., in air. The same oocysts chain could then be photographed after flooding with 1:1 glycerin:water as was done in the original description (Clopton et al., 1991). Digital photographs were taken with a Nikon Coolpix 995 camera fitted with an Optem 25-70-14 adapter (www.optemintl.com). Photographs were converted to grayscale, and brightness and contrast were adjusted slightly using Adobe Photoshop 6.0. The plate of figures was assembled using Adobe Illustrator 10.

Figure 1 is from the original species description and is a drawing of oocysts suspended in 1:1 glycerin:water. In this kind of preparation, oocysts can move freely, so they can be seen in various profiles. Under these conditions, *G. niphandrodes* oocysts are remarkably uniform in size and shape, with a raised center portion and flared ends as seen from the side, and an oblong, almost rectangular shape but with rounded corners and an apparent sheath holding the chain together (Fig. 2). At the same magnification, but without flooding (Fig. 3), the raised equatorial portion is evident, and the oocysts have a truncated diamond shape when viewed ''from above,'' that is, so that maximum length and width were apparent. There is no evidence that sputter-coating and photographing the oocysts without prior use of standard SEM preparation techniques (glutaraldehyde and osmium fixation, dehydration, critical point drying) altered overall shape. The profile as seen from the side is well preserved in the SEM specimens (Figs. 5, 6); additional features not obvious at the light level are the regularly spaced longitudinal ridges of almost uniform height, width, and spacing on either side of the central raised area. However, the original description drawings hint at these ridges (Fig. 1, labeled 4A in the original description), and they can be seen, although not clearly, in the glycerin preparations (Figs. 1, 2, arrows). Immersion in glycerin:water evidently makes a sheath of some kind visible, perhaps by swelling it, thus giving the oocysts a more

rectangular appearance then when dry (cf. Figs. 1, 2 vs. Fig. 3). The broad, truncated diamond shape seen in dry oocysts is also clearly seen in the SEMs (Figs. 3, 4). There was no ultrastructural evidence of an enclosing external sheath holding the oocysts in a chain. Oocyst ends were flared slightly, and the chain itself was twisted with adjacent oocysts offset slightly from one another.

There are 2 main contributions of this study. First, the demonstration that oocyst structure as seen under the light microscope is preserved through sputter-coating and scanning electron microscopy, even though standard fixation, dehydration, and critical-point drying are not performed on the specimens. Second, there are distinct, fine folds or ridges, arranged in a distinctive pattern, on the oocysts. Although these structural features are potentially useful in future gregarine systematic work, such use requires comparative information on oocyst surface architecture from a variety of gregarine taxa.

The differences between oocyst structure as seen in glycerin:water suspensions versus in air at oil immersion magnifications could be the result of refraction of the light beam, although there is also a possibility that the cysts are contained within a sheath of some kind that is expanded in glycerin:water. These differences also emphasize the importance of reporting oocyst preparation methods in detail, for example, as exemplified by Clopton et al. (2004), when using such measurements in taxonomic studies.

The authors wish to thank Kit Lee for assistance with the scanning electron microscopy.

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Helminths of Hudsonian Godwits, *Limosa haemastica*, From Alaska and Manitoba

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ABSTRACT: In total, 21 Hudsonian godwits, *Limosa haemastica* (Charadriiformes: Scolopacidae), were examined for helminths, 10 from Bristol Bay, Alaska, and 11 from Churchill, Manitoba. Seventeen species of helminths (9 trematodes, 6 cestodes, 2 nematodes) were collected, but only 1 trematode species, *Plagiorchis elegans*, was found in common between the 2 sample sites. All 17 species are new records for this host and 2 cestodes, *Capsulata edenensis* and *Malika limosa*, are new records for North America. In general, both prevalence and intensities were low, and species richness ranged from 1 to 6 (mean = 2.4). Most of the differences in the helminth faunas between the 2 sites were attributed to difference in habitats, freshwater in Manitoba versus saltwater in Alaska.

The Hudsonian godwit, *Limosa haemastica* (Linnaeus, 1758) (Charadriiformes: Scolopacidae), is a large shorebird that breeds in 5 disjunct areas in North America, including 2 distinct sites along Hudson Bay (1 in Ontario, the other in Manitoba), the Arctic Coast of the Northwest Territories, the southern coast of Alaska, and the west coast of Alaska, and winters in southern South America, especially in Argentina (Elphick and Klima, 2002). This species is found in the boreal forest–tundra transition zone and breeds in areas where open sedge meadows intermix with forests. During staging and migration, godwits feed primarily on invertebrates in coastal tidal flats.

Although the helminths of its Palearctic congeners, the black-tailed godwit, *Limosa limosa* (Linnaeus, 1758) and the bar-tailed godwit, *L. lapponica* (Linnaeus, 1758), have been comparatively well studied (Sergienko, 1972; Schmidt and Allison, 1989; Kornyushin and Greben, 2000; Piersma et al., 2001), almost nothing is known about the helminths of *L. haemastica*. The description of *Wardium villocirrosus* (= *Hymenolepis villocirrosus*) from the Hudsonian godwit in Alaska by Deblock and Rausch (1967) appears to be the only published record of a helminth from this host.

Ten Hudsonian godwits were collected between 13 and 24 June 1991, and a single godwit on 19 June 1992 by ASD under scientific permit numbers WS-M54 and WS-M26 issued by the Canadian Wildlife Service. All birds were collected in the vicinity of the Twin Lakes (58°39′24″N, 93°51′05″W) in the Cape Churchill Wildlife Management Area in Manitoba, Canada. The area is freshwater habitat consisting of scattered clumps of black spruce and tamarack forest, sedge meadows, and lichen heaths. Between 23 and 29 July 2001, 10 Hudsonian godwits were collected by A.G.C. and J.M.K. from a 6.0-km-long section of littoral zone just north of the mouth of the Egegik River, Bristol Bay, Alaska, between Bishop Creek (58°14′31″N, 157°29′43″W), and Big Creek (58°17′01″N, 157°32′5″W).

All hosts were killed with a shotgun, and each was examined within 6 hr of collection. In the Manitoba birds, only the lower intestinal tract (small intestine, ceca, large intestine, cloaca) was examined. In the Alaska birds, all internal organs were examined. The koilin of the ventriculus was removed, and both the ventriculus and proventriculus tissues were teased apart. Skin and blood were not examined in either sample. Nematodes were studied in temporary mounts of lactophenol, and trematodes and cestodes were stained with either Meyer's carmine or Ehrlich's hematoxylin, cleared in methyl salicylate, and mounted in Canada balsam. Voucher specimens were deposited in the National Parasite Collection, Beltsville, Maryland under accession numbers 99447–99463.

In total, 17 species of helminths (9 trematodes, 6 cestodes, 2 nematodes) were recovered from 21 godwits (Table I). All are new host records for *L. haemastica*. Godwits from Alaska were infected with 9 species (5 trematodes, 2 cestodes, 2 nematodes), and those from Manitoba were also infected with 9 species (5 trematodes, 4 cestodes), but only 1 trematode species, *Plagiorchis elegans*, was present in birds at both sites. Four godwits from Alaska and 3 from Manitoba (7 of 21) were negative for helminths. In general, prevalence and intensities were low (Table I), and species richness ranged from 1 to 6 species per host (mean = 2.4 ± 1.2 SD).

Two cestode species, *Capsulata edenensis* and *Malika limosa*, are

TABLE I. Helminths of *Limosa haemastica* from Alaska and Manitoba.

	Alaska (n = 10)			Manitoba (n = 11)		
		Intensity			Intensity	
	Prevalence (%)	Mean	Range	Prevalence (%)	Mean	Range
Trematoda						
Austrobilharzia variglandis (1)*	10	4	—	—	—	—
Tanaisia fedtschenkoi (2)	20	21	1–40	—	—	—
Plagiorchis elegans (3)	10	4	—	27	3	1–4
Pachytrema paniceum (4)	10	2	—	—	—	—
Brachylecithum lari (5)	10	1	—	—	—	—
Echinoparyphium recurvatum (3)	—	—	—	55	3	1–10
Echinostoma trivolvis (3)	—	—	—	18	2	2–3
Cotylurus cornutus (3)	—	—	—	27	3	1–5
Lacunovermis macomae (3)	—	—	—	9	500	—
Cestoda						
Capsulata edenensis (3)	10	1	—	—	—	—
Ophryocotyle proteus (3)	30	80	8–208	—	—	—
Echinocotyle minutilla (3)	—	—	—	17	4	1–7
Echinocotyle tenuis (3)	—	—	—	9	1	—
Malika limosa (3)	—	—	—	9	1	—
Nadejdolepis sp. (3)	—	—	—	9	1	—
Nematoda						
Viktorocara limosae (6)	30	6	1–15	NE†	—	—
Viktorocara capillaris (6)	20	31	1–60	NE†	—	—

* Location in host: (1) blood vessels, (2) kidneys, (3) small intestine, (4) gall bladder, (5) bile ducts, (6) Koilin lining.
† Not examined.

recorded for the first time from North America and both may be godwit specialists. Sandeman (1959) described *C. edenensis* from *Limosa lapponica* from Scotland, and Fuhrmann (1907) described *M. limosa* (synonym *Dilepis limosa*) from *Limosa limosa* from Europe. All of the remaining species appear to be generalists, recorded from a variety of shorebirds and waterfowl (e.g., Wong and Anderson, 1991; Canaris and Kinsella, 2000, 2007).

The disparity between the helminth fauna of the Alaska and Manitoba godwits may not be as dramatic as it appears, because only the lower gastrointestinal tract was examined in the Manitoba sample, making the presence or absence of the 2 species of gizzard nematodes (*Viktorocara limosae*, *V. capillaris*) and 4 species of extraintestinal trematodes (*Austrobilharzia variglandis*, *Tanaisia fedtschenkoi*, *Pachytrema paniceum*, *Brachylecithum lari*) problematic. Nevertheless, there was no overlap in the intestinal tapeworm fauna, and only 1 species of intestinal trematode (*Pl. elegans)* was found in both samples. These differences are probably related to the difference in the food habits of the birds, primarily feeding in freshwater habitats in Manitoba, and feeding on tidal flats in Alaska. In particular, the most prevalent cestode from Alaska, *Ophryocotyle proteus*, is primarily associated with marine habitats.

Both *O. proteus* and *C. edenensis* appear to be cosmopolitan helminths of godwits, having been reported from *L. lapponica* in areas as far apart as the Netherlands (Piersma et al., 2001) and New Zealand (Schmidt and Allison, 1989), and now in *L. haemastica* from Alaska and Manitoba.

Although both sample sizes were small, the Hudsonian godwit appears to have a relatively small parasite fauna. Seven of 21 birds (33%) were negative for helminths, and 9 of 17 helminth species were found in only 1 bird. The highest prevalence for any species was *Echinoparyphium recurvatum* at 54%, and mean intensities of infection were generally low. Mean species richness was only 2.4, possibly indicating a narrow specialization in food habits, because most of its parasites require an invertebrate intermediate host. Nothing is known about the parasites of this bird during its migration along the eastern coast of North America, or on its wintering grounds on the east coast of South America. These areas should be the subjects of future investigations.

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First U.S. Record of the Hard Tick *Ixodes* (*Pholeoixodes*) *gregsoni* Lindquist, Wu, and Redner

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ABSTRACT: *Ixodes* (*Pholeoixodes*) *gregsoni* Lindquist, Wu, and Redner, a species of hard tick described in 1999 in Canada, was recovered from a harvested fisher (*Martes pennanti* Erxleben) and a domestic cat (*Felis silvestris catus* Ragni and Randi) in Vermont in 2001 and from harvested mink (*Mustela vison* Schreber) in Maine in 2003. These samples are the first records of this species within the United States. Although knowledge of this tick's natural history and distribution are still preliminary, these records indicate a possible greater distribution for *I. gregsoni* than initially perceived. Although its status as a disease vector is presently unknown, natural resource professionals should be aware of the possibility of this tick's occurrence in the northeastern United States.

To date, 13 species of hard ticks have been found in Maine (Keirans and Lacombe, 1998), whereas Vermont records 12 species (Nielsen, 1998). Species of *Ixodes* found in both states include *I. angustus* Neumann, *I. cookei* Packard, *I. marxi* Banks, *I. muris* Bishopp and Smith, and *I. scapularis* Say (Smith et al., 1992; Nielsen, 1998). *Ixodes gregsoni* Lindquist, Wu, and Redner was recently described from specimens of harvested mustelids inhabiting boreal forests in Ontario and eastern Canada (Lindquist et al., 1999). Here, we report the first U.S. record of this tick collected from Maine and Vermont.

Both Vermont and Maine passively survey for tick species through the use of tick identification and submission programs (Smith et al., 1992; Nielsen, 1998). Submissions from the general public, physicians, and veterinarians have enabled investigators to map the geographic distribution of ticks from both states while documenting new records (Keirans and Lacombe, 1998; Nielsen, 1998). As a result of this surveillance program in Vermont, in April 2001, 2 female *I. gregsoni* were removed from a domestic cat, *Felis silvestris catus* Ragni and Randi, in Barre (Washington County), Vermont (RML 123624). In December 2001, a single larva of *I. gregsoni* was removed from a harvested fisher, *Martes pennanti* Erxleben, in Morrisville (Lamoille County), Vermont (RML 123621) (Fig. 1). This discovery led Maine investigators to solicit tick submissions from licensed furbearer trappers in Maine. In November 2003, 2 female *I. gregsoni* were recovered from 2 harvested mink, *Mustela vison* Schreber, from Smyrna (Aroostook County), Maine (RML 123622-23) (Fig. 1). Ticks were tentatively identified as *I. gregsoni*, based on the descriptions provided by Lindquist et al. (1999). Specimens thought to be *I. gregsoni* were submitted to the U.S. National Tick Collection, Institute of Arthropodology and Parasitology, Georgia Southern University, Statesboro, Georgia, for species confirmation.

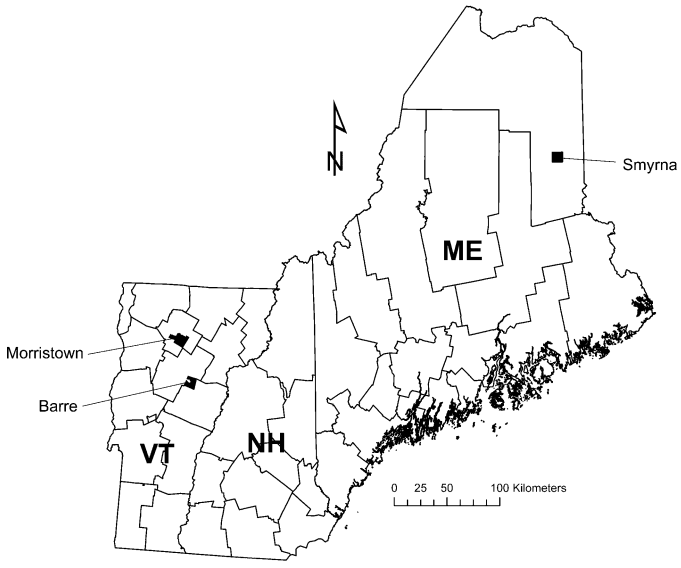

FIGURE 1. Locations of towns in Maine and Vermont where *Ixodes gregsoni* was collected.

This report documents the presence of a newly described species of tick in the United States. In describing this species, Lindquist et al. (1999) reported *I. gregsoni* from mustelids in boreal forests in Ontario and eastern Canada. In Maine and Vermont, however, 1 Vermont submission (RML 124624) was found in a peridomestic setting, whereas Maine specimens were retrieved from wetland habitats.

Morphologically, *I. gregsoni* closely resembles *I. texanus* Banks (Lindquist et al., 1999), a tick that parasitizes mustelids and members of the Procyonidae (raccoons) (Cooley and Kohls, 1945). Although the 2 species are thought to be closely related, sharing characteristics such as humps along the anterior margin of the basis capituli, a key morphological difference found between female *I. gregsoni* and *I. texanus* is a subtriangular, larger porose area on the basis capitulum in *I. gregsoni* (Lindquist et al., 1999). Because much information about this tick remains to be investigated, how closely related the 2 species are is presently unknown. The relatedness of these 2 species, determined through molecular techniques, should be a focus of future research. To date, *I. texanus* has not been recorded from either Maine or Vermont (Nielsen, 1998), despite reports of its collection in New York (Durden and Keirans, 1996). The hosts for *I. texanus* and *I. gregsoni* (raccoons and mustelids) are widespread in both states. Previous work detecting the presence of tick-borne diseases in mustelids (Main et al., 1979) also raises the question of *I. gregsoni* as a possible vector for transmission of an arbovirus such as Powassan encephalitis in New England. Future studies should examine this tick for Powassan and other tick-borne agents, because the primary vector of Powassan, *I. cookei*, also is found on mustelids (Main et al., 1979). Natural resource professionals and wildlife managers should be aware of the possible presence of this tick where appropriate hosts exist in the northeastern United States.

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Ixodes philipi (Acari: Ixodidae): Phylogenetic Status Inferred From Mitochondrial Cytochrome Oxidase Subunit I Gene Sequence Comparison

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ABSTRACT: *Ixodes philipi* ticks were collected from the nest burrows of streaked shearwaters, *Calonectris luecomelas*, on 3 different islands of Japan (Awashima: 38°45′N, 139°24′E; Mikurajima: 33°52′N, 139°36′E; and Omorijima: 36°8′N, 133°10′E). The mitochondrial cytochrome oxidase subunit I (COI) gene sequence was determined for each tick. The COI sequences of 9 other ixodid tick species also were determined, and they were used for taxonomic positioning of *I. philipi*. A metastriata tick, *Amblyomma triguttatum*, was used as an outgroup reference for the analysis. Phylogenetic examination indicated that the *I. philipi* ticks are on the branch with *Ixodes turdus* and *Ixodes acutitarsus* weakly, and the bootstrap value of this branching was low. Three different analyses, maximum parsimony, genetic distance, and maximum likelihood, support this conclusion. To further refine this analysis, 2,761 base pairs (bp) of sequence, which included the genes for t-RNA^{Met}, NADH dehydrogenase subunit 2 (ND2), tRNA^{Trp}, tRNA^{Cys}, tRNA^{Tyr}, and COI, were determined and compared for 6 *I. philipi* ticks from the 3 different collection sites. Although a base substitution (T to C in the ND2 gene for an Awashima tick) and 2 transitions (G to A in the COI gene for 1 Omorijima tick) have occurred, the overall sequences were highly conserved. Preserved mitochondrial sequences in the ticks from 3 widely separated locations suggest the possibility of gene flow, which was probably accomplished by migratory seabirds.

Ixodes philipi was described by Keirans and Kohls (1970) based on partially fed female ticks obtained from streaked shearwaters, *Calonectris luecomelas*. Since then, no additional data regarding the tick have been published, because of its rare occurrence in Japan. Recently, *I. philipi* were collected from *C. leucomelas* or from soil of their nest burrows, or both. Detailed morphology of the ticks and their host relationships have been published previously (Takahashi et al., 2005); this tick species seems to solely depend on seabirds as hosts. Studies in Japan have demonstrated the presence of ixodid ticks on birds (Saito et al., 1974; Uchikawa and Saito, 1983; Miyamoto et al., 1993); however, none of these studies sufficiently documented the ixodid fauna on seabirds.

Calonectris leucomelas occurs on the islands surrounding the Japanese archipelago; 86 breeding islands have been recognized to date (Oka, 2004). The largest island is Mikurajima, where the bird population is roughly estimated at 1.8–3.5 million. One of us (M.T.) has surveyed various localities and demonstrated that *I. philipi* are found exclusively in *C. leucomelas* nests and that these ticks feed on these seabirds. Ticks were collected from Mikurajima Island (33°52′N, 139°36′E), in the Pacific Ocean and from Awashima Island (38°45′N, 139°24′E) and Omorijima Island (36°8′N, 133°10′E) in the Sea of Japan. All unfed adult and nymphal ticks were subjected to mitochondrial DNA (mtDNA) sequencing.

Phylogenetic analysis using nuclear ribosomal RNA, internal transcribed spacer sequences, or both, have provided consistent tick species identification (McLain et al., 1995; Crampton et al., 1996; Zahler et al., 1997; Fukunaga et al., 2000). mtDNA also was used successfully to assess the phylogeography of the related ticks *Ixodes scapularis* (Norris et al., 1996) and *Ioxdes pacificus* (Kain et al., 1999). mtDNA has a higher mutation rate than do nuclear genes because of its maternal inheritance and haploid nature. It has been used to differentiate morphologically similar and synonymized tick species (Murrell et al., 1999). We therefore determined the nucleotide sequence for mitochondrial cytochrome oxidase subunit I (COI) gene, the most conserved gene of tick mitochondrial proteins, but a gene that has diverged enough to distinguish between tick species. We used the results quantitatively to establish the phylogenetic status of *I. philipi* within ixodid tick species and to assess the relationships between the ticks found at geographically distant islands.

Ixodes philipi were collected from the nest burrows of *C. leucomelas* in each study area: Mikurajima Island on 6 June 2004 (1 female and 1 nymph); Awashima Island on 22 August and 21 September 2004 (1 male for each collection date); and Omorijima Island on 3 July 2004 (2 males). A nymph of *Ixodes vespertilionis* that had fed on *Rhinolophus cornutus* was collected on 23 April 2005 at a mining gallery in Uenomura village, Gunma, Japan. An unfed adult of *I. acutitarsus* was collected at Shimokita, Aomori in 2002 by flagging. *Ixodes asanumai* (laboratory-reared), *Ixodes granulatus* (Kagoshima; flagged), *Ixodes monospinosus* (Saitama; flagged), *Ixodes nipponensis* (Chungdu, Korea; flagged), *Ixodes ovatus* (Hokkaido; flagged), *Ixodes pavlovskyi* (laboratory-reared), *I. turdus* (Yamagata; had fed on a bird), and *Amblyomma triguttatum* (Brisbane, Australia; flagged) had all been stored in alcohol. The other tick sequences were all from databases (DDBJ/EMBL/GenBank). Tick species, collection site, and collectors are as indicated in Table I.

Live males, females, or nymphs were individually crushed in preparation for extracting genomic DNA. Ticks preserved in alcohol were shaken in 500 μl of saline-EDTA for 5 min (0.15 M NaCl and 0.1 M EDTA, pH 8.0), air-dried, and then used for DNA extraction. DNA extraction was carried out as described previously (Fukunaga et al., 2000). For polymerase chain reaction (PCR) amplification, 5 μl of solution (approximately 10 ng of DNA) was used. A primer set for amplification of the COI gene was chosen in the tRNA^{Trp} gene (forward primer, 221R5-Trp; 5′-cta tgt atc ctt caa agt aca a-3′) and COII gene sequence region (reverse primer, IxoCO2R; 5′-gaa tga tca tga aaa aaa att att-3′) based on previously published sequences (Shao et al., 2005). To amplify larger fragments, including the genes for tRNA^{Met}, ND2, tRNA^{Trp}, tRNA^{Cys}, tRNA^{Tyr}, and COI, a forward primer based on internal sequence of the tRNA^{Ile} gene was used (IphiIleF2; 5′-aat gcc tga aaa agg gtt atc ttg-3′). The COI region was amplified as described previously (Fukunaga et al., 2000). The target DNA was amplified in a 50-μl reaction mixture containing 10 ng of template DNA, 200 μM each deoxyribonucleotide triphosphate, the primer set at a concentration of 1 μM, 1.5 units of *Taq* polymerase (ExTaq, Takara shuzo, Kyoto, Japan), and ExTaq reaction buffer. The reaction was subjected to 30 thermal cycles consisting of 94 C for 60 sec, 45 C for 60 sec, and 68 C for 120 sec with a GeneAmp PCR System 9700 (Applied Biosystems,

TABLE I. Species of ticks studied.

Species	Subgenus	Location of collection	Collector	Accession no.
Prostriate ticks				
Ixodes acutitarsus	*Ixodes*	Aomori, Japan	H. Fujita	AB105166
Ixodes asanumai	*Ixodes*	Lab strain, Tokyo, Japan	F. Hayashi	AB231674
Ixodes granulatus	*Ixodes*	Kagoshima, Japan	H. Fujita	AB231673
Ixodes hexagonus	*Pholeoixodes*		Database	AF081828
Ixodes holocyclus	*Sternalixodes*		Database	AB075955
Ixodes monospinosus	*Ixodes*	Kanagawa, Japan	M. Nakao	AB231672
Ixodes nipponensis	*Ixodes*	Chungdu, Korea	Y. Sato and K. H. Park	AB231671
Ixodes ovatus	*Partipalpiger*	Hokkaido, Japan	M. Nakao	AB231670
Ixodes pavlovskyi	*Ixodes*	Hokkaido, Japan	M. Nakao	AB231669
Ixodes persulcatus	*Ixodes*		Database	AB073725
Ixodes philipi	*Scaphixodes*	Awashima, Niigata, Japan	M. Takahashi	AB231663
		Mikurajima, Tokyo, Japan	M. Takahashi	AB231665
		Omorijima, Tottori, Japan	M. Takahashi	AB231666
Ixodes ricinus	*Ixodes*		Database	AY945447
Ixodes turdus	*Ixodes*	Saitama, Japan	K. Fujimoto	AB231668
Ixodes uriae	*Ceratoixodes*		Database	AB087746
Ixodes vespertilionis	*Eschatocephalus*	Gunma, Japan	M. Takahashi	AB231667
Metastriate ticks				
Amblyomma triguttatum		Queensland, Australia	R. Shao	AB113317

Osaka, Japan). DNA synthesis was extended to 180 sec for the larger fragments described above. The PCR product targeting the whole COI gene gave a 1.8-kilobase (kb) fragment, and the larger fragments were about 3 kb.

Amplified DNA fragments were directly sequenced in both directions by using custom-synthesized primers. Sequencing primers for each tick species were made individually every 400–500 nucleotides. Sequencing reactions were carried out as described previously (Shao et al., 2004). The sequences determined here and the sequences for other tick species in databases were aligned with ClustalX (Thompson et al., 1997). For both pairwise and multiple alignments, the gap opening penalty was 15.00, and the gap extension penalty was 6.66. For multiple alignments, the delay divergent sequence was 30%, and the DNA transition weight was 0.50. Neighbor-joining (NJ), maximum likelihood (ML), and maximum parsimony (MP) trees were constructed with PAUP 4.0 (Swofford, 2000). The general time-reversible model and gamma-distributed rates (Lanave et al., 1984) were used in ML tree construction; the instantaneous rate matrix, base frequencies, and the shape of gamma distribution were estimated by PAUP. Bootstrap tests (1,000 replicates) were run on the NJ and the consensus MP trees (Saito and Nei, 1987). DDBJ/EMBL/GenBank accession numbers (AB231663–74) of the sequences are all indicated in Table I.

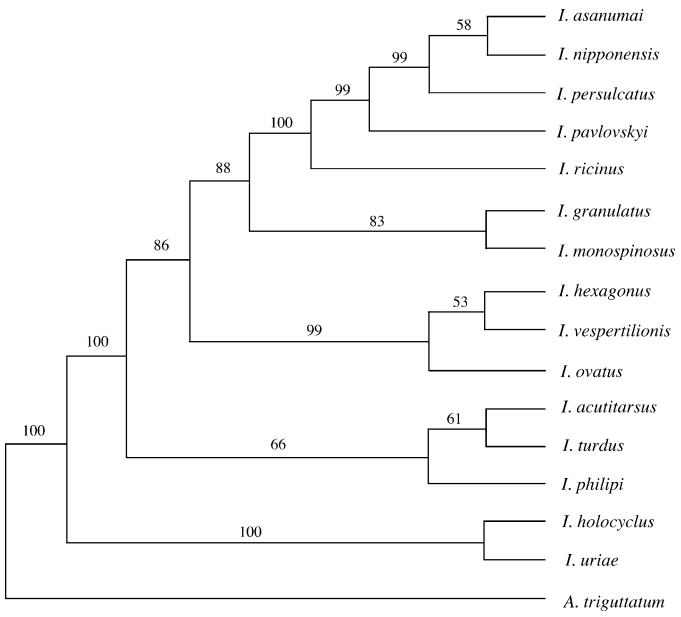

FIGURE 1. Phylogenetic relationship of the ixodid tick taxa as estimated from cytochrome oxidase subunit I (COI) gene sequences. The tree was constructed using COI sequences. The percentage of bootstrap support (1,000 replicates) is shown above each branch. See Table I for the full name of each species. The COI sequences from the ticks used in this tree are as follows: *Ixodes acutitarsus*, *I. asanumai*, *I. granulatus*, *I. hexagonus*, *I. holocyclus*, *I. monospinosus*, *I. nipponensis*, *I. ovatus*, *I. pavlovskyi*, *I. persulcatus*, *I. philipi*, *I. ricinus*, *I. turdus*, *I. uriae*, *I. vespertilionis*, and *Amblyomma triguttatum*.

The nucleotide sequences were determined to quantitatively assess the phylogenetic divergence of the ixodid tick taxa. The sizes of the entire COI sequences from the 12 ixodid tick species are 1,539 nucleotides, except for *I. holocyclus* (1,542 nucleotides) and *I. uriae* (1,545 nucleotides); the entire sequence was not available for *I. ricinus*. The COI gene for *A. triguttatum* was 15 nucleotides shorter than that of the other species. Phylogenetic analyses of the COI sequences of ticks produced an NJ tree, an ML tree, and a MP tree. These trees were largely congruent with each other, and thus only the MP tree is shown in Figure 1. *Ixodes philipi* was clustered with *I. acutitarsus* and *I. turdus*, both classified into different subgenera (Clifford et al., 1973). Bootstrap values did not securely support this branching. Moreover, the grouping using COI sequences does not concur with the tick phylogeny that has been proposed based on morphological aspects (Yamaguti et al., 1971; Clifford et al., 1973). Additional molecular data for the other tick species are needed to establish phylogenetic status. *Ixodes persulcatus* is widely distributed throughout the taiga forests from eastern Europe to Japan, and *I. ovatus* is found throughout Japan. Both tick species frequently feed on rodents and shrews (Nakao and Miyamoto, 1993; Nakao et al., 1994). In contrast, *I. philipi*, which is thought to be exclusively associated with seabirds, is a rare tick species (Takahashi et al., 2005) that has been insufficiently characterized.

Prostriate ticks have the same arrangements of their mitochondrial genes (Black and Roehrdanz, 1998; Shao et al., 2004); PCR indicated that *I. philipi* ticks have this same gene organization (data not shown). Whereas the COI sequence is the most conserved in tick mitochondrial

TABLE II. Comparison of the signature nucleotides of *Ixodes philipi* ticks from Awashima, Mikurajima, and Omorijima islands.

Source of ticks		Position of mitochondrial genes		
		830 (ND2)	261 (COI)	805 (COI)
Awashima Island	A	T	G	G
	B	C (Ile to Thr)	G	G
Mikurajima Island	A	T	G	G
	B	T	G	G
Omorijima Island	A	T	A (synonymous)	A (Ala to Thr)
	B	T	G	G

protein genes, NADH dehydrogenase subunit (ND)2, ND4L, and ND6 genes are highly diverged (Shao et al., 2004). We, therefore, examined about 3 kb of the DNA segment, including the genes of $tRNA^{Met}$, ND2, $tRNA^{Trp}$, $tRNA^{Cys}$, tRNATyr, and COI, for ticks collected from the different localities to examine their geographical divergence. The 2,761 nucleotide sequences, beginning at $tRNA^{Met}$ gene and ending by the COI gene, were compared with those of 6 ticks from 3 different collection sites (2 sequences of the ticks from Awashima and Omorijima islands were not deposited in databases, because their sequences are identical with that of the tick from Mikurajima Island). The sequences of 4 tRNA genes were highly conserved, and no nucleotide substitution has occurred. In the ND2 gene sequence (972 nucleotides), only 1 base substitution (T to C at nucleotide position 830) was found in an adult tick from Awashima Island, and the mutation resulted in an amino acid substitution of isoleucine to threonine (Table II). In general, the mutation rate in ND2 gene is much higher than that of the COI gene; however, there were 2 nucleotide changes in COI of ticks from Omorijima Island. A transition at nucleotide position 261 (G to A) is synonymous, and another transition at nucleotide position 805 (G to A) altered the amino acid sequence, changing alanine to threonine.

Our results indicate that there is a high degree of genetic similarity among the 3 *I. philipi* geographic populations that were analyzed; the data do not support a low rate of divergent evolution or a significant isolation-by-distance effect. Mitochondrial protein sequences such as ND2 gene are generally rapidly evolving than the faster bases in ribosomal RNA sequences (Simon et al., 1994). *Ixodes philipi* seemed to have the potential for natural, long-range dispersal, because these ticks parasitize seabirds. Shearwaters are highly mobile and undertake long, complex migrations; thus, it seems likely that there could be significant levels of gene flow between tick populations in the northern Pacific and within the Japan Sea areas. Although further population genetic study is needed to support this hypothesis, information regarding the mitochondrial DNA sequences of these parasitic ticks could significantly contribute to our understanding of seabird ecology.

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Cryptosporidiosis in Patients with Colorectal Cancer

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ABSTRACT: Parasitological examination of feces was carried out for 55 patients with diagnosed colorectal cancer before chemotherapy. Except for *Cryptosporidium* sp., no other intestinal parasites were found in the specimens; moreover, only the patients with watery diarrhea were *Cryptosporidium* sp.–positive by enzyme immunoassay. Prevalence of infection in the group of patients with diarrhea (23 persons) was 43.5%, whereas it was 18% for the entire group of patients under study. Coproantigens of this parasite were detected primarily in the patients with tumors located on the left side (in the sigmoid and descending colon).

In Poland, colorectal cancer (CRC) is the second most commonly occurring malignant tumor; it is also the second leading cause of death from cancer among both men and women (Sobczak et al., 2005). The risk of developing this tumor increases with age, reaching its peak in the eighth decade of life. Research results demonstrate that CRC development is qualified by genetic and/or environmental factors. The hereditary form accounts for only a small portion of CRC cases and is related to Lynch syndrome or familial adenomatous polyposis (Lynch and Smyrk, 1999; Abdel-Rahman and Peltomaki, 2004). A link has also been established between occurrence of CRC and mutations in several genes, e.g., p53, NF1, APC, and K-Ras (Gonciarz et al., 2004; Bertholon et al., 2006). A diet rich in red meat and animal fat on the one hand, and poor in vitamins and calcium on the other, represents a significant CRC factor. In contrast, a high-fiber, calcium-rich diet prevents the disease. In addition to improper dietary habits, a low level of physical activity is considered to be another CRC risk factor (Kesse et al., 2005; Lim et al., 2005).

On the basis of previous research, the effect of parasitic infection on the process of carcinogenesis cannot be ruled out. For example, the association of *Schistosoma* spp. infection and bladder cancer, hepatocellular carcinoma, and colorectal cancer is well documented based on results of epidemiological and experimental studies (Ishii et al., 1994; Qiu et al., 2005). It is also widely recognized that CRC, like other tumors, is linked to immune deficiencies, which promote opportunistic invasion, including symptomatic cryptosporidiosis. So far, only few papers have been published on cryptosporidiosis in patients with reduced immunity (Tanyuksel et al., 1995; Sreedharan et al., 1996; Majewska et al., 1999). The aim of this work was to estimate the frequency of *Cryptosporidium* sp. infection in patients with CRC (with and without diarrhea).

The study included 55 patients (25 women and 30 men) with colorectal cancer. The average age of the patients was 64.7 yr. Among the patients, 23 had watery diarrhea. Most of the patients, 43 persons (78.2%), had a tumor on the left side (in the wall of the descending colon, sigmoid colon, or rectum); the remaining 12 patients (21.8%) had a right-sided CRC (in the wall of the caecum, ascending colon, or transverse colon) (Tables I, II). This difference was statistically significant (chi-square test, $P < 0.0001$).

The patients under study exhibited various degrees of histological differentiation, as well as various pathologic stages. A low histologic grade, G1, represents a well-differentiated tumor, while G2 indicates a moderately differentiated tumor, and G3 represents a poorly differentiated tumor. Low and high histologic grades may be associated with the independent outcome of the stage group for both colon and rectal adenocarcinoma. To describe the pathologic stage, we used the Astler–Coller classification of cancer histotypes: A, with infiltration limited to the mucosa; B, with infiltration to the deeper layers of the intestinal wall; C, spreading to the lymph nodes; and D, with distant metastases (Tables I, II). All the investigated tumors were adenocarcinomas.

Statistical comparisons between groups of infected patients with left- and right-sided CRC were performed using Fisher's exact test. To evaluate the correlation between *Cryptosporidium* sp. infection and the stage of tumor development, as well as the degree of its histological differentiation, the chi-square test was applied.

Parasitological examination of feces was carried out before chemotherapy to eliminate the effect of the cytostatic treatment on the risk of any infection. For this purpose, the material was sampled within 2 days after the colonoscopy resulting in cancer diagnosis. Apart from direct coproscopic examination, an enzyme immunoassay, ProSpecT® *Cryptosporidium* Microplate Assay (Alexon Trend, Lenexa, Kansas), was used for detection of *Cryptosporidium* sp. coproantigens.

Microscopic examination of fecal specimens collected from the CRC patients did not reveal any cysts or eggs of parasites. Pathomorphological examinations did not indicate *Cryptosporidium* sp. in histological sections of the tumors.

The enzyme immunoassay showed that fecal specimens of 18.2% of the 55 CRC patients, both with and without diarrhea, were *Cryptosporidium* sp.–positive. Considering patients only with diarrhea (23 CRC patients), the percentage of positive tests was 43.5%. The frequency of

TABLE I. Number of patients with CRC depending on tumor location and cancer histotype.

Cancer histotype	Left-sided location	Right-sided location	Total
A	2	0	**2**
B	20	5	**25**
C	8	4	**12**
D	11	3	**14**
X*	2	0	**2**
Total	**43**	**12**	**55**

* X = lack of histological assessment.

TABLE II. Number of patients with CRC depending on tumor location and differentiation grade.

Differentiation grade	Left-sided location	Right-sided location	Total
G1	3	0	**3**
G2	30	10	**40**
G3	5	2	**7**
X*	5	0	**5**
Total	**43**	**12**	**55**

* X = lack of an assessment of differentiation grade.

Cryptosporidium sp. infection in left-sided tumor patients (9/43 = 21%) and right-sided tumor patients (1/12 = 8%) was not significantly different (Table III). Among the left-sided tumors, *Cryptosporidium* sp. infection affected mostly those with tumors located in the sigmoid colon and descending colon. No *Cryptosporidium* sp.–specific coproantigens were detected in the patients with an early stage of cancer (A) or with its least aggressive form (G1). Data presented in Table III suggest that *Cryptosporidium* sp.–positive assays were observed mainly in the patients with stage B of cancer and grade 2 (G2) of malignancy. However, statistical analysis did not reveal the significant association between the frequency of *Cryptosporidium* sp. infection and the degree of histological differentiation or the stage of clinical cancer development.

Opportunistic parasites, including *Cryptosporidium parvum*, are frequently detected in patients with immunodeficiency syndrome, as well as those suffering from cancer (Wong, 1984; Rudrapatna et al., 1997; Ballal et al., 1999; Orenstein and Dieterich, 2001; Baqai et al., 2005). To date, no studies have been carried out on cryptosporidiosis in CRC patients. Our observations, which included CRC patients with or without acute diarrhea, have demonstrated that 18% of assays were *Cryptosporidium* sp.–positive. This percentage is high if compared with the results in other studies (Sreedharan et al., 1996; Radrapatna, 1997), which found that, respectively, 1.3% and 0.3% of patients with cancer and diarrhea after chemotherapy were affected by cryptosporidiosis. Moreover, it is commonly known that chemotherapy represents an additional factor in promoting symptomatic opportunistic infections. Within the group of CRC patients with watery diarrhea, we found cryptosporidiosis in 43.5% of the patients. Such a high frequency of infection is comparable with results of studies on AIDS patients, more than 40% of whom were found to be infected by *C. parvum* (Ballal et al., 1999; Baqai et al., 2005).

The high prevalence of *Cryptosporidium* sp. infections in our patients suggests that these protozoans are widespread in the environment. The importance of wild rodents as reservoir hosts and sources of infection for local human communities in Poland has been demonstrated by Sińnski (1993), Bajer et al. (2002), and Bednarska et al. (2003). The contamination of the environment, e.g., water and soil, with *Cryptosporidium* sp. is from oocysts that are excreted with feces of many species of livestock and wild animals. Long-term viability of oocysts in the environment results from their high resistance to chemicals and changes in temperature and pH (Fayer et al., 1998; Gasser and O'Donoghue, 1999). An increased interest of researchers in the pathogen has been observed since the outbreak of waterborne cryptosporidiosis in humans in the United States (MacKenzie et al., 1994) and because of the deaths of HIV-positive patients as a result of *C. parvum* invasion (Colford et al., 1997).

A high prevalence of infections by opportunistic parasites among patients with cancer may result from their reduced immunity; on the other hand, however, a possible role of the parasites in the etiopathogenesis of cancer cannot be ruled out. Epidemiological and experimental studies have established evident connection between liver cancer, colon cancer, and *Schistosoma japonicum* infection, as well as between urinary bladder cancer and *Schistosoma hematobium* infection (Ishii et al., 1994). These authors view the carcinogenic properties of these parasites as high. In addition, Oliveira et al. (1997) described a correlation between colon cancer and Chagas disease caused by *Trypanosoma cruzi*, whereas Lee et al. (2002), in a histological examination of a tumor mass of the colon, detected eggs of *Enterobius vermicularis*. Leroy et al. (1994–1995) proposed that colon cancer invasion may be promoted by adhesion of *Entamoeba histolytica* to the epithelium. According to these authors, molecules released by trophozoites during the early stage of adhesion disturb intercellular junctions, which may promote metastasis of cancer cells.

The role of *Cryptosporidium* sp. in CRC development cannot be ruled out. Sasahara et al. (2003) demonstrated reduced apoptosis of intestinal epithelial cells following *C. parvum* infection using a mouse model. This protozoan is usually an intracellular parasite of the small intestine epithelium; however, histological examinations of biopsies have also revealed the parasite's presence outside cells, near the colonic wall (Orenstein and Dieterich, 2001).

It is difficult to explain the higher frequency of *Cryptosporidium* sp. infections in patients with left-sided CRC, as well as in those with tumors without metastasis. We hope that further studies carried out on a larger group of patients will resolve this issue.

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TABLE III. Frequency of *Cryptosporidium* infection in patients with right- and left-sided locations of CRC.

	Number of patients with A–D cancer histotypes/number of infected patients					Number of patients with G1–G3 differentiation grade/number of infected patients				Total of examined patients/number of infected patients
	A	B	C	D	X*	G1	G2	G3	X*	
Left-sided location	2/**0**	20/**4**	8/**2**	11/**2**	2/**1**	3/**0**	30/**7**	5/**1**	5/**1**	43/**9**
Right-sided location	0	5/**1**	4/**0**	3/**0**	0	0	10/1	2/0	0	12/**1**
Total	2/**0**	25/**5**	12/**2**	14/**2**	2/**1**	3/**0**	40/**8**	7/**1**	5/**1**	55/**10**

* X = lack of an assessment of disease stage and differentiation grade.

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DNA Probes for Identifying Chromosomes 5, 6, and 7 of *Schistosoma mansoni*

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ABSTRACT: *Schistosoma mansoni* has a genome of 270 Mb contained on 8 pairs of chromosomes. C-banding has been a useful technique in identifying the 7 autosomal and sex chromosomes. However, even with C-banding, *S. mansoni* chromosomes 5, 6, and 7 are difficult to discriminate from each other, because of their small sizes, morphological similarity, and poor banding patterns. We have identified probes that specifically paint chromosomes 5, 6, and 7 of *S. mansoni* with the use of chromosome microdissection and the degenerate oligonucleotide-primed polymerase chain reaction (DOP-PCR). Exact chromosome identification is required for accurate chromosome mapping of genomic clones and genetic elements, which is an essential component of the schistosome genome project.

Schistosomiasis is a debilitating parasitic disease caused by several species of *Schistosoma*. An estimated 200 million people are currently infected and 500–600 million more people are at risk of exposure to infection in 75 countries worldwide (Engels et al., 2002). The current reliance on a single drug, praziquantel, as treatment necessitates the development of new antischistosomal control strategies. Schistosome genomics offers the best prospects for developing new targets for drugs and vaccines, improving diagnostics, and dissecting the biological basis for host–parasite interactions (El-Sayed et al., 2004). *Schistosoma mansoni* has a genome of approximately 270 Mb that is 34% G + C (Simpson et al., 1982). The genome is approximately 40% repetitive and 60% represents single copy or small gene families (Simpson et al., 1982). For *S. mansoni*, the genetic information is contained on 8 pairs of chromosomes—7 autosomal pairs and 1 sex pair (Short, 1983). Chromosomes contain between 15 and 64 Mb of DNA and can be distinguished by differences in size, arm ratios, and amount of heterochromatin (C-banding pattern) (Hirai and LoVerde, 1995). It is estimated that the *S. mansoni* genome contains between 15,000 and 20,000 genes (Franco et al., 2000).

Among species of *Schistosoma*, cytogenetic analysis of chromosomes 5, 6, and 7 is difficult for the nonexperienced, because these chromosomes are of small size, are morphologically similar, and have poor banding patterns (Hirai et al., 2000). Easy and reliable identification of chromosomes 5, 6, and 7 would be an advance for chromosome mapping for the schistosome genome project (e.g., Tanaka et al., 1995; Le Paslier et al., 2000; LoVerde et al., 2004), as well as an aid for establishing systematic relationships with other schistosomes such as *S. japonicum*. Chromosome microdissection is a useful technique to obtain specific probes from a specified region of chromosomes (e.g., Behrens

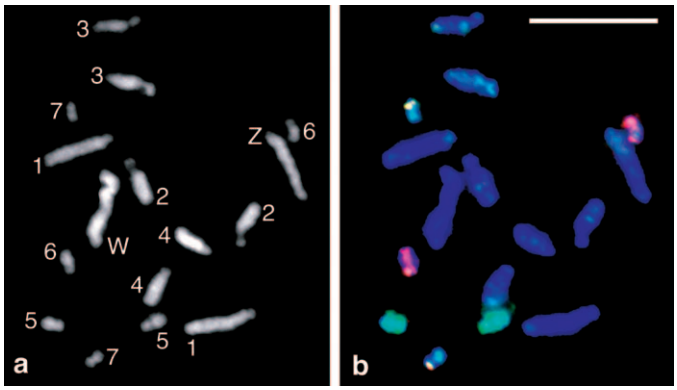

FIGURE 1. A 3-color FISH image of metaphase chromosomes of *Schistosoma mansoni* painted by microdissection probes obtained from chromosomes 5, 6, and 7. Left and right panels show (**a**) DAPI-stained and (**b**) FISH images, respectively. Green FISH signals identify chromosome 5, pink for chromosome 6, and yellow for chromosome 7. Note that chromosomes 5, 6, and 7 are easily distinguished from the other chromosomes in the complement by the specific chromosome color. Scale is 10 μm.

et al., 1997; Taguchi et al., 2003). To obtain convenient probes for studies of schistosome chromosome evolution, but also to contribute to schistosome genomics, we adapted the methods of chromosome microdissection and DOP-PCR for the schistosome parasites.

In the present study, we report the identification and use of painting probes for chromosomes 5, 6, and 7 of *S. mansoni* with the use of chromosome microdissection followed by DOP-PCR. Chromosome preparations were made with *S. mansoni* (NMRI) sporocysts from *Biomphalaria glabrata*-infected snails as previously described (Hirai and LoVerde, 1995; Hirai and Hirai, 2004). Metaphase spreads for microdissection and fluorescence in situ hybridization (FISH) were prepared on 24 × 60-mm coverslips and glass slides, respectively. After identifying chromosomes with the use of morphological diagnostic markers described in previous studies (Hirai and LoVerde, 1995; Hirai and Hirai, 2004), 8–20 whole-chromosome metaphase preparations for each chromosome were scraped with a chromosome microdissection technique (Taguchi et al., 1997, 2003). In brief, glass needles with about 1–2-μm-diameter tips were produced from glass capillaries (GD-1, Narishige, Tokyo, Japan) with the use of a pipette puller, PC-10 (Narishige). Microdissection was then performed under an inverted microscope (Olympus, Tokyo, Japan) equipped with a mechanical micromanipulator, Eppendorf 5171 (Hamburg, Germany). The scraped chromosome fragments were placed into a 0.5-ml tube under a binocular microscope. DOP-PCR was used to amplify DNA of the scraped chromosome segments in a thermal cycler, PTC-100 (MJ Research, Boston, Massachusetts). The procedure used was essentially the same as reported by Christian et al. (1999), with minor modifications. PCR was performed in a final volume of 10 μl, containing 1.0 μl Thermo Sequenase DNA polymerase (Amersham Biosciences Corporation, Bath, United Kingdom); 1.0 μl Thermo Sequenase reaction buffer; 200 μM of each dATP, dCTP, dGTP, and dTTP; and 4 μM DOP primer (5′-CCG ACT CGA GNN NNN NAT GTG G-3′). Thirty microliters of mineral oil were added to prevent evaporation. The thermal profile consisted of 95 C for 10 min, 6 cycles of 94 C for 1 min, 30 C for 2 min, and a ramp of 0.1 C/sec up to 65 C for 3 min, 30 cycles of 94 C for 1 min, 56 C for 1 min, and 72 C for 3 min, followed by a single 72 C for 5 min; then the samples were held at 4 C until removal. To verify DNA recovery from the PCR, electrophoresis for each sample was run on a 1.5% agarose gel at 100 V for 1 hr. When the appropriate sized products (300–800 base pairs) were obtained, a hapten of biotin- or digoxigenin-labeled nucleotide was incorporated into the DNA segments by the 2nd-generation DOP-PCR with 2 μl of the 1st-generation products used as a template. The 50-μl labeling reaction for the 2nd PCR contained 20 U Thermo Sequenase DNA polymerase; 26 mM Tris-HCl, pH 9.0; 6.5 mM $MgCl_2$; 200 μM of each dATP, dCTP, dGTP, and dTTP; 40 μM biotin-16-dUTP (Roche Applied Science, Tokyo, Japan) or digoxigenin-11-dUTP (Roche Applied Science); and 4 μM DOP primer. The thermal profile consisted of 95 C for 5 min, 25 cycles at 94 C for 1 min, 56 C for 1 min, and 72 C for 3 min, followed by 72 C for 5 min; then the samples were held at 4 C until removal.

FISH with the biotin- or digoxigenin-labeled probes was performed as previously described (Taguchi et al., 1997). For 3-color FISH, the 3 microdissection-regenerated probes were mixed in the following ratio: mixture of biotin- and digoxigenin-labeled chromosome 7 DNA fragments (1.5 part and 1 part, respectively):digoxigenin-labeled chromosome 6 DNA fragments:biotin-labeled chromosome 5 DNA fragments (1:1:1). The haptens, biotin, and digoxigenin, were detected with FITC-avidin (Vector Laboratories Inc., Burlingame, California) and Rhodamin antidigoxigenin antibody (Roche Applied Science), respectively. Consequently, chromosome 5 is stained as a greenish color, chromosome 6 as pinkish, and chromosome 7 as yellowish (Fig. 1). The hybridized chromosomes were observed under an Olympus BX-50 fluorescence microscope. FISH images of suitable metaphases were acquired and stored with a cooled charge-couple device (Photometrics-Seki Technotron, Tokyo, Japan) equipped with the M-FISH system.

The karyotype of *S. mansoni*, 14 autosomes and 2 (W and Z) sex chromosomes, have been studied in detail (Hirai et al., 2000). Poor banding patterns (only C-banding is available) of the parasite chromosomes, a problem for invertebrate chromosomes in general, was an issue in the detailed cytogenetic analysis (Hirai et al., 2000; Taguchi et al., 2000). This was especially true for *S. mansoni* chromosomes 5, 6, and 7, as they are very similar to each other.

We employed the powerful technique of chromosome microdissection to obtain specific probes for schistosome chromosomes 5, 6, and 7 in order to discriminate them from each other. As shown in Figure 1, a comparison of the DAPI (diamidino-2-phenylindole) stained and chromosome paintings of FITC, Rhodamin, and the mixture of FITC and Rhodamin fluorescent colors revealed that each painting probe is able to discriminate the 3 chromosomes, i.e., green for chromosome 5, pink for chromosome 6, and yellow for chromosome 7. In the present study, each chromosome stained by the 3 different colors was identified with the use of the morphology of chromosomes counterstained with DAPI by a skilled author (H.H.). Confusion regarding chromosomes 5, 6, and 7 can now be resolved with the use of these probes. In addition, chromosome painting probes are also being developed for chromosomes of other species in the genus of *Schistosoma*. Whether those developed for *S. mansoni* will identify the chromosomes for other schistosome species is yet to be determined.

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